THE CHROMAFFIN GRANULE MEMBRANE

by

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I declare that the work presented in this thesis, except where stated, was performed entirely by myself in the Department of Biochemistry, University of Edinburgh Medical School, Teviot Place, Edinburgh between October 1975 and September 1978.

SUMMARY

1. Chromaffin granule membranes have been prepared from bovine adrenal medullae. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of the membranes, in the presence of dithiothreitol, revealed 66 bands ranging in molecular weight c 150,000 to 10,000 daltons. The majority of the bands were minor staining components.

2. The granule membranes also contain several glycoproteins (ten) which could be stained in gels of the membranes by a variety of methods (the periodic acid - Schiff, dansyl hydrazine and fluorescein isothiocyanate conjugated concanavilin A and wheat germ agglutinin stains and tritiation with [^{3}H]-borohydride following periodate or galactose oxidase treatment of the membranes).

3. The organization of the protein components of the membrane has also been investigated by labelling or digesting intact granules or broken membranes with the following reagents: lactoperoxidase /[^{125}I]iodide as a reagent for tyrosine residues; N-(iodoacetylaminoethyl)-5-napthylamine-1-sulphonic acid as a reagent for cysteine residues; pronase; galactose oxidase/[^{3}H]-borohydride; and periodate/[^{3}H]-borohydride. Penetration of the membranes by the reagents was assessed by examination of the labelling of intra-granular proteins.

4. The majority of the granule membrane polypeptides could be labelled when intact granules were treated with the impermeant reagents. Relatively few polypeptides could only be labelled when broken membranes were used. In contrast, the carbohydrate moieties of the glycoproteins appear to be exposed only on the matrix side of the membrane.

5. Of the enzyme activities of the membrane phosphatidylinositol kinase, NADH: (acceptor) oxidoreductase and Mg^{++} activated adenosinetriphosphatase are exposed on the outside of the granule. Dopamine-β-hydroxylase, the

major protein species of the granule membrane, appears to be exclusively located on the matrix side of the membrane. The second major protein, chromomembrin B, spans the membrane and is exposed at both surfaces.

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Abbreviations

aaa	aromatic amino acid decarboxylase (E.C.4.1.1.28)
ADR	adrenaline
AMP-PCP	adrenylyl (βγ-methylene) diphosphonate
ANS	analinonapthelene sulphonic acid
ATPase	adenosine 5'-triphosphatase
CBB	coomassie brilliant blue
Con A	concanavilin A
DβH	dopamine-β-hydroxylase (E.C.1.14.17.1)
DA	dopamine
DH	dansyl hydrazine
DIDS	4,4'-diisothiocyano-2,2'-stilbene disulphonic acid
DOPA	3,4-dihydroxyphenylalanine
DSA	diazotized sulphanilic acid
DTT	dithiothreitol
FITC	fluorescein isothiocyanate
FMMP	formylmethionylmethyl phosphate
f.t.	freeze-thawing
G	granules
GM	granule membrane
GO	galactose oxidase
GP	glycoprotein
hepes	4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid
1,5-I-AEDANS	N-(iodoacetylaminoethyl)-5-napthylamine-1-sulphonic acid
IAI	isethionyl acetimidate
LP	lactoperoxidase
1,5-Me-AEDEANS	N-(mercaptoethanolacetylaminoethyl)-5-napthylamine-1-sulphonic acid

Mg^{++} ATPase	Mg^{++} activated adenosine 5'-triphosphatase (E.C.3.6.1.4)
NA	noradrenaline
P_2 granules	see P44 for preparation
PAS	periodic acid-Schiff
PBS	phosphate buffered saline
PCMB	p-chloromercuribenzoate
PER	periodate
PNMT	phenylethanolamine-N-methyl transferase (E.C.2.1.1.28)
RCA	ricinus communis agglutinin
RT	room temperature
SAM	S-adenosyl methionine
SDS	sodium dodecyl sulphate
SITS	4'-isothiocyanostilbene-2,2'-disulphonic acid
Stains-all	(1-ethyl-2-[3-(1-ethylnapthol[1,2d]thiazolin-2-ylidene)-2-methylpropenyl]-napthol 1, 2d-thiazolium bromide
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TH	tyrosine hydroxylase (E.C.1.14.16.2)
TNBS	trinitrobenzene sulphonate
WGA	wheat germ agglutinin

All other abbreviations used are in accordance with the instructions given by the Biochemical Journal to prospective authors ('The Biochemical Journal - Policy of the Journal and Instructions to Authors' revised 1978 edition).

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CHAPTER 1

INTRODUCTION

INTRODUCTION

1.1 THE CHROMAFFIN SYSTEM

1.1.1. Introduction

The chromaffin granule is the specialized organelle of chromaffin cells responsible for the uptake, synthesis, storage and release of the catecholamine class of hormones (Kirshner, 1974). The function of chromaffin cells is the release of catecholamines into the blood. This is achieved by 'stimulus-secretion' coupling (Douglas, 1968). Acetylcholine, released from the splanchnic nerve, depolarises the chromaffin cell membrane, an action potential is evoked, calcium ions enter the cell and catecholamines are released by exocytosis.

Chromaffin cells, as defined by Coupland (1965), develop from neuro-ectoderm, are innervated by pre-ganglionic sympathetic nerve fibres (the splanchnic nerve), can synthesize and secrete catecholamines and store them in sufficient concentrations to be stained with chrome salts. This affinity for chrome salts led to the term 'chromaffin' being coined (Kohn, 1900, 1902, 1903). Chromaffin cells occur in invertebrates and vertebrates (Coupland, 1972). The largest group of chromaffin cells in mammals is found in the medulla of the suprarenal (adrenal) gland. This gland, which is located on top of the kidney, is composed of two distinct parts - the outer cortex, responsible for the synthesis and secretion of steroid hormones, and the inner medulla, which synthesizes and secretes catecholamines. The cortex is of mesodermal origin and is thought to exert some kind of hormonal influence over the medulla (Holtzbauer & Sharman, 1972).

The chromaffin system can be used as a model for other secretory systems which utilise specialized storage granules and also for sympathetic neurons,which store and secrete noradrenaline. The chief

advantage of using chromaffin cells as an experimental system lies in the ease with which large quantities of chromaffin granules can be purified (usually from the bovine adrenal medulla). Because of this chromaffin granules have been extremely well characterized and can be used to investigate problems associated with secretion from granules. These problems, as summarized by Apps and Phillips (1979), are:-

1. How are the secretory proteins identified within the cell?
2. How and where are granules formed?
3. How are granules transported to their eventual release site?
4. How are small molecules stored within granules?
5. How is the secretory stimulus transmitted to the release site?
6. What is the biochemical basis of the release process?
7. What is the ultimate fate of secretory granules after secretion?

Not all of these problems are best suited to solution in the chromaffin system, for instance the first two questions are being answered by experimentation in the endocrine pancreas (Blobel and Dobberstein,1975; Meldolesi,1974). However, the fourth and sixth of these problems can be readily studied in chromaffin granules.

1.1.2 The Adrenal Medulla as an Experimental System

Most of the biochemical and physiological studies have been performed on the bovine adrenal gland. Subcellular fractionation techniques are the most commonly employed methods for studying the biochemistry of the adrenal medulla. Chromaffin granules and their membranes have been purified (see later). Granule preparations usually comprise both noradrenaline and adrenaline containing granules. Resealed ghosts of chromaffin granules have also been prepared (Phillips, 1974a). The plasma membrane (Nijjar and Hawthorne, 1974; Wilson and Kirshner, 1976) and golgi apparatus (Trifaro' and Duerr, 1976) have only been partially purified. Preparations of adrenal medullary mitochondria are usually heavily contaminated with cortical mitochondria since it is very dif-

ficult to dissect all the cortex away from the medulla. Possible post-mortem artefacts should also be borne in mind.

1.1.3 Ultrastructure of the Adrenal Medulla

The adrenal medulla is composed of chromaffin cells, connective tissue, blood vessels and nerve fibres. Two kinds of chromaffin cell can be distinguished when sections are fixed with glutaraldehyde and stained with osmium tetroxide - one containing granules with only slightly electron dense cores and the second type containing granules whose cores are very electron dense. The latter type of granule contains noradrenaline and the former adrenaline (Coupland, 1965).

The chromaffin cell is polarized. At one end the cell is innervated (the nerve end) and at the opposite side the cell is separated from the blood capillary by the basement membrane. Secretion tends to take place at the surface nearest the capillary and this is reflected in the high concentration of granules here (although granules are distributed around the whole of the cell periphery). At the nerve end of the cell lie the nucleus, golgi apparatus and rough endoplasmic reticulum. Mitochondria are scattered throughout the cytoplasm. The adrenal medullary cells also contain lysosomes and microtubules.

1.1.4 Ultrastructure of Chromaffin Granules

Chromaffin granules are seen in the electron microscope as round, membrane bound vesicles with an electron dense core. Bovine granules are approximately 3,400Å in diameter (Burack et al.,1962) and there are 1 - 2,000 granules per cell. The granule membrane appears to be typically trilamellar and is separated from the core by an electron transparent zone of about 150A (D'Anzi, 1969).

1.1.5 Purification of Chromaffin Granules

Chromaffin granules can be purified from homogenates of adrenal

medullae in isotonic sucrose by differential centrifugation (Blaschko and Welch, 1953; Hillarp et al., 1953) followed by either continuous (Phillips, 1973) or discontinuous (Smith and Winkler, 1967a; Helle et al., 1971; Schneider, 1972) sucrose density gradient centrifugation. Highly purified granules can be obtained in isosmotic media by the use of Ficoll/deuterium oxide gradients (Trifaro' and Dworkind, 1970). These procedures produce a mixture of noradrenaline and adrenaline containing granules. Pure noradrenaline granules can be obtained, though in very low yield, by centrifugation through 2.2 M sucrose (Winkler, 1969).

1.1.6 Composition of Chromaffin Granules

For the composition of bovine chromaffin granules see Table 1.1. The granules are extremely rich in protein (Hillarp, 1959) giving them a greater density than mitochondria and this is the basis of their purification. As well as catecholamines (Blaschko and Welch, 1953; Hillarp et al., 1953) the granules contain substantial amounts of nucleotides (Hillarp, 1958a) and lipid (Hillarp, 1959) of which phospholipid and cholesterol are the major components (Blaschko et al., 1967; Winkler et al., 1967). Trace amounts of calcium (Borowitz et al., 1965; Borowitz, 1967; Borowitz, 1969; Serck-Hanssen and Christiansen, 1973), magnesium (Borowitz et al., 1965), ascorbate (Terland and Flatmark, 1975) and mucopolysaccharides (Fillion et al., 1971) are also present.

72% of the catecholamine content of bovine granule preparations is adrenaline and 27% noradrenaline (Eade, 1958). Traces of dopamine (Eade, 1958) were shown to be present by comparing the distribution of dopamine with that of the other catecholamines following sucrose density gradient centrifugation of a crude granule fraction. This is the normal procedure for identifying trace substances as components of chromaffin granules.

Constituent	% of dry weight	μmol/mg protein	Approximate concentration in matrix
Protein	42		200 mg/ml
Adrenaline	13.7	1.8	700 mM
Noradrenaline	5.2	0.68	
Dopamine	0.2	0.02	
ATP	16.8	0.56	220 mM
ADP		0.09	
AMP		0.02	
GTP		0.07	
GDP + UDP		0.06	
Phospholipids	15	0.48	0
Cholesterol	4.5	0.29	0
Muco-polysaccharides	0.6:2.2	MW not known	MW not known
Calcium	0.12	0.076	30 mM
Magnesium	?	0.02	6 mM
Ascorbic acid	0.5	0.063	15 mM

Table 1.1 Composition of Bovine Chromaffin Granules

Data for this Table has been compiled from Tables 1 and 2 of Apps and Phillips (1979) and Table 2 of Winkler (1976).

ATP comprises 70% of the nucleotides present with 10% ADP and 2.5% AMP (Hillarp and Thieme, 1959; Goetz et al., 1971) although the proportion of each is species dependent. It has been suggested that in vivo ATP represents the bulk of the nucleotides and that ADP and AMP arise by enzymic hydrolysis during isolation. There is an ATPase present in the membrane. Other nucleotides have been shown to be present (GTP, 9.2%; GDP and UDP 6.9%; see Goetz et al., 1971).

Nucleotide content of chromaffin granules is usually expressed as the molar catecholamine to nucleotide ratio. This is usually about 4.5 (Hillarp, 1958b; Banks, 1965; Smith and Winkler, 1967a; Trifaro' and Dworkind, 1970). However, in certain human phaeochromocytomas this ratio may be as high as 35 (Winkler and Smith, 1972). Nascent granules contain nucleotides but are catecholamine deficient (Winkler and Smith, 1975; Viveros et al., 1971; O'Brien et al., 1972), so there may be no significance in the ratio 4.5 (see later).

1.1.7 The Granule Lysate

Dilution of chromaffin granules with a large volume of hypotonic medium will lyse them. Insoluble material (the granule membrane) can be removed by ultracentrifugation. The soluble fraction (the granule lysate) contains all the catecholamines and nucleotides (Hillarp and Nilson, 1954) of the granule together with 77% of the protein (Hillarp, 1958c), 64% of the calcium (Borowitz, 1967) and at least some of the mucopolysaccharides (Baumgartner et al., 1974).

The soluble proteins comprise a family of acidic proteins, the chromogranins, and the enzyme dopamine-β-hydroxylase (Blaschko et al., 1967; Winkler, 1976). Sodium dodecyl sulphate polyacrylamide gel electrophoresis, which separates proteins according to their molecular weight, reveals several bands one of which comprises 40% or more of the soluble

proteins (Schneider et al., 1967; Smith and Winkler, 1967b; Smith and Kirshner, 1967). This major band has been termed chromogranin A (Schneider, et al., 1967). The slowest moving band is dopamine-β-hydroxylase (Smith et al., 1970; Hörtnagel et al., 1974).

Chromogranin A has been purified by DEAE-cellulose and Sephadex G-200 chromatography (Helle, 1966; Smith and Kirshner, 1967; Smith and Winkler, 1967b). The amino acid composition (Helle, 1966; Smith and Winkler, 1967b; Smith and Kirshner, 1967; Foldes et al., 1973; Aunis et al., 1975b) reveals a high content of acidic amino acids and is similar to the amino acid composition of total soluble proteins (Strieder et al., 1968; Helle and Serck-Hanssen, 1969). The molecular weight of chromogranin A has been variously estimated in the range 79,000 - 81,200 daltons (Smith and Winkler, 1967b; Kirshner and Kirshner, 1969; Helle, 1971b). However, there is disagreement as to whether chromogranin A is a glycoprotein (Smith and Winkler, 1967b; Winkler and Smith, 1975; Aunis et al., 1975b; Geissler et al., 1977; Huber et al., 1979; Cahill and Morris, 1979). Specific antisera to chromogranin A has been prepared and shown to cross react with other chromogranins (Hörtnagl et al., 1974). This has led to suggestions that the smaller chromogranins are proteolytic fragments of chromogranin A. A study of the physical properties of purified chromogranin A indicates a configuration approaching that of a random coil polypeptide (Smith and Winkler, 1967b; Kirshner and Kirshner, 1969).

Dopamine-β-hydroxylase comprises 5% of the soluble proteins of the chromaffin granule (Hörtnagl et al., 1974). It is also found in the granule membrane where it is the major protein constituent (Hörtnagl et al., 1972). Approximately equal activities are found in the lysate and membrane (Belpaire and Laduron, 1968; Winkler et al., 1970; Helle, 1971a; Schneider, 1972). The significance, if any, of this dual

location is unknown. Membrane bound and soluble DβH appear to be identical with respect to amino acid composition, electrophoretic mobility and immunological properties (Hörtnagl et al., 1972; Foldes et al., 1972; Kuzuya and Nagatsu, 1972). Various procedures have been used to purify DβH (Kaufman and Friedman, 1965; Goldstein et al., 1972; Foldes et al., 1972; Rush et al., 1974; Aunis et al., 1975a; Aunis et al., 1973). The enzyme has a molecular weight of 290,000 daltons (Kaufman and Friedman, 1965) and comprises 4 x 75,000 dalton subunits (Wallace et al., 1973; Craine et al., 1973; Aunis et al., 1974 ; Kirshner, 1974). Its amino acid composition is significantly different from that of chromogranin A and the rest of the chromogranins (Hörtnagl et al., 1972; Foldes et al., 1973; Craine et al., 1973; Wallace et al., see Winkler, 1976; Ljones et al., 1976). DβH is a glycoprotein but there is disagreement as to the sugars present. In one case (Wallace et al., 1973) mannose, N-acetylglucosamine and to a lesser extent fucose were found whereas Aunis et al. (1974) detected galactose and N-acetylneuraminic acid.

1.1.8 The Relationship between Chromogranin A and Dopamine-β-Hydroxylase

Helle (Helle, 1971a, 1971b; Helle and Serck-Hanssen, 1975) claims that chromogranin A is a subunit of DβH. This is based upon her finding of immunological cross reactivity between chromogranin A and DβH. Aunis et al. (1974) also claim a similarity in amino acid composition between these two proteins prepared from bovine adrenal glands. The molecular weight of chromogranin A is also very similar to that of DβH in its reduced form. However, there appears to be an overwhelming body of evidence against this view: the mobilities of these two peptides are different in phenol-acetic acid-urea gels; other workers (see earlier) have found different amino acid compositions for DβH and chromogranin A;

N—cetylpyridinium chloride will precipitate all the chromogranins, including chromogranin A, but not DβH (Hörtnagl et al., 1972); only DβH binds concanavilin A (Rush et al., 1974; Aunis et al., 1975a); and other workers have not found any immunological cross reactivity between DβH and chromogranin A (Sage et al., 1967; Hörtnagl et al., 1974). It has been suggested that the preparation of chromogranin A used by Helle was contaminated with traces of DβH, which is an excellent antigen, and that Aunis et al. (1974) did not completely separate DβH and chromogranin A.

1.1.9 The Granule Membrane

The granule membrane is recovered as a brown pellet after ultracentrifugation of lysed chromaffin granules. The membranes can be further purified by washing away last traces of lysate (Winkler et al., 1970) and also by sucrose density gradient centrifugation (Schneider, 1972; Phillips, 1973; Helle, 1973a; Muller and Kirshner, 1975), which will separate granule membranes from mitochondrial membranes. Chromaffin granule membranes are extremely light due to their low protein to lipid ratio compared to other membranes (Winkler et al., 1970; Schneider, 1972). Only the myelin sheath contains less protein.

Granule membranes contain all the phospholipid (however, see later) and cholesterol of the granule (Winkler et al., 1970; 1972a) as well as about a fifth of the protein (Hillarp, 1958c; Winkler et al., 1966; Taugner, 1971a; Schneider, 1972). The membranes contain characteristically high amounts of cholesterol (Winkler et al., 1970; Schneider, 1972) and the unusual phospholipid lysolecithin (Blaschko et al., 1967b; Winkler et al., 1967; Da Prada et al., 1972b). Because of the ability of the latter to fuse membranes (Howell and Lucy, 1969) it has been implicated in exocytosis (see later).

All the known enzymes of the chromaffin granule, apart from the soluble form of DβH, are localised within the membrane. These are phosphatidylinositol kinase (Phillips, 1973, Muller and Kirshner, 1975), Mg^{++}-activated ATPase (Banks, 1965) and an NADH:(acceptor) oxidoreductase (Flatmark et al., 1971). The membrane also contains a b type cytochrome (Banks, 1965; Flatmark et al., 1971) and possibly a flavoprotein (Flatmark et al., 1971).

Silsand and Flatmark (1974) have purified cytochrome b-561 and determined its amino acid composition. They have also shown it to be made up of eight subunits each of molecular weight 4380 daltons and to contain protohaem IX. No function is known for cytochrome b-561 but it has been postulated that together with NADH: (acceptor) oxidoreductase and the flavoprotein it forms a mini respiratory chain supplying electrons to DβH, a mixed function oxidase.

The Mg^{++}-activated ATPase has been partially purified by lubrol PX solubilization of granule membranes followed by ion-exchange chromatography (Trifaro' and Warner, 1972) and by velocity sedimentation on glycerol gradients following solubilization with nonidet P42 or by extraction of membrane lipids with dichloromethane (Apps and Reid, 1977). The molecular weight of the enzyme is thought to be in the region 400,000 daltons (Apps and Reid, 1977) and is probably involved in proton translocation (Bashford et al., 1976; Casey et al., 1976, 1977). The pH of the matrix of the granule is about 5.5 (Johnson and Scarpa, 1976a, b; and others). The formation of a pH gradient and membrane potential may be concerned with catecholamine and/or nucleotide transport across the granule membrane.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis of granule membranes according to Winkler (1971) reveals two major chara-

cteristic staining bands which were termed chromomembrin A and B. Chromomembrin A has been identified as the membrane bound form of DβH (Hörtnagl et al., 1972). Chromomembrin B is not seen in gels run by Kirshner (1974), although the electrophoretic conditions and gel percentage were different. Bartlett (see Winkler, 1976) reports a molecular weight of 28,000 daltons for chromomembrin B which has been partially purified by solubilization with SDS followed by chromatography on Sephadex G-200 (Hörtnagl et al., 1971). Antibodies raised against chromomembrin B have been used to confirm its location in chromaffin granulesand absence in other adrenal medullary membranes (Hörtnagl et al., 1973).

1.1.10 The Presence of Lipid in the Matrix

Mylroie and Koenig (1971) have isolated lipoproteins from chromaffin granules by triton X-100 treatment and ultrasonication followed by centrifugation. This results in 82% of the phospholipid and 68% of the protein remaining in the supernatant. They claim that even without triton X-100 treatment or ultrasonication 40 - 65% of the protein and 30 - 61% of the phospholipid of the granule is soluble.

Helle (1968) has also claimed that the soluble proteins of the chromaffin granule are lipoproteins containing 0.2 - 0.4 μmole protein bound phosphate but this is, apparently,not lipid solvent extractable (Helle, 1971b).

Winkler et al. (1972a) has repeated Mylroie and Koenig's (1971) experiments in which untreated lysed granules were subjected to centrifugation. They found that after a 30 minute centrifugation at 180,000 g 13.3% of the phospholipid remained in the supernatant but that further centrifugation reduced this to 1.4%. Thus it would appear that the presence of soluble phospholipid found by Mylroie and Koenig (1971) is due

to solubilization with triton X-100 or by sonication and the application of insufficient centrifugal forces.

It has been suggested that lipoproteins exist in the matrix but are pelleted by centrifugation and hence recovered with the membrane (Helle, 1973a, b). This view is not supported by perfusion experiments where significant amounts of phospholipid are not recovered after stimulation of the adrenal gland despite the presence of all the expected soluble components of the granule in the perfusate (Schneider et al., 1967). It is also known that granule lipid has a very low turnover rate whereas the opposite would be expected if lipids were secreted (Winkler et al., 1972).

1.1.11 The Biosynthesis of Catecholamines

For the synthesis of catecholamines from tyrosine see Fig. 1.1. This subject has been reviewed by Kirshner (1975).

Biosynthesis of catecholamines is controlled by feedback inhibition of tyrosine hydroxylase (E.C.1.14.16.2.) by catecholamines. This enzyme is a mixed function oxidase requiring a reduced pteridine electron donor (possibly tetrahydrobiopterin) and is probably located in the cytoplasm. 3, 4-dihydroxyphenylalanine (DOPA) is decarboxylated to dopamine by a broad specificity pyridoxal phosphate requiring aromatic amino acid decarboxylase (E.C.4.1.1.28.). Dopamine-β-hydroxylase (E.C.1.14.17.1.), another mixed function oxidase, is the only biosynthetic enzyme present in the granule. It is a copper containing glycoprotein and requires its substrate (dopamine) to be transported into the granule before hydroxylation to noradrenaline can occur. The natural electron donor for the enzyme is unknown but is probably ascorbate since this is the best donor and is also found in granules.

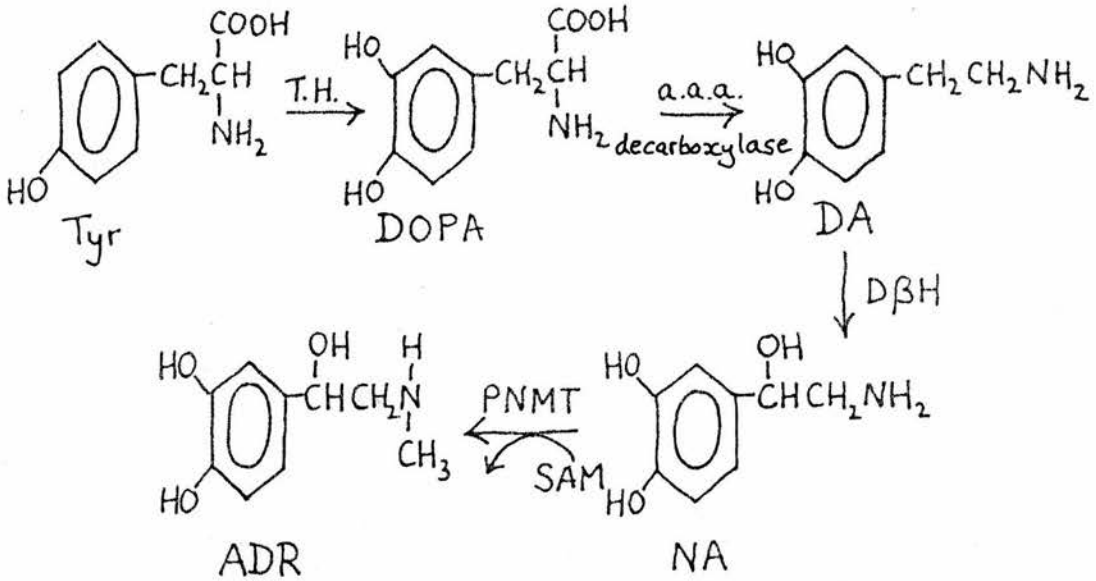


Fig. 1.1 The Biosynthesis of Catecholamines

TH, tyrosine hydroxylase; a.a.a. decarboxylase, aromatic amino acid decarboxylase; DβH, dopamine-β-hydroxylase; PNMT, phenylethanolamine N-methyl transferase. Tyrosine hydroxylase, aromatic amino acid decarboxylase and phenylethanolamine N-methyl transferase are cytoplasmic enzymes whilst dopamine-β-hydroxylase is located in the chromaffin granule.

In adrenaline containing cells an extra cytoplasmic enzyme is present - phenylethanolamine N-methyl transferase (E.C.2.1.1.28.) which transfers methyl groups from S-adenosylmethionine to noradrenaline to form adrenaline. However, to do this noradrenaline must pass out of the granule into the cytoplasm. After methylation, adrenaline is transported back into the granule. It has been suggested, although there is no evidence, that dopamine can first be methylated and then transported into the granule where the epinine would be hydroxylated by DβH which is capable of performing this reaction.

1.1.12 The Storage of Catecholamines

Over 90% of the catecholamine content of chromaffin cells is stored in granules (Smith and Winkler, 1972). This store is maintained by the relative impermeability of the membrane to catecholamines, the existence of a storage complex for catecholamines, and active transport of catecholamines across the membrane. The granule membrane is permeable to catecholamines but is sufficiently impermeable to allow active transport to accumulate high concentrations of catecholamines against a concentration gradient. The storage of 0.7 M catecholamine and 0.22 M nucleotide requires some form of interaction to lower the osmotic pressure. It is known that at $0^{o}C$ little or no catecholamine and ATP is lost from the granule (Falck et al., 1956) but exogeneous catecholamine is free to enter and leave granules without mixing with the endogeneous store (Hillarp, 1959; Kirshner et al., 1966). At higher temperatures catecholamines leak out (Hillarp, 1958c) and exogeneous catecholamines enter the granule and are free to mix with the endogenous store (Kirshner et al., 1966; Taugner and Hasselbach, 1966). This indicates that the complex is less stable at high temperatures. It is presumably stablized by active transport of catecholamines back across the membrane.

1.1.13 The Nature of the Storage Complex

The complex is not covalently linked since lysis of the granules produces individual components. Separate constituents of the granule lysate are also recovered in perfusates of stimulated adrenal glands. Catecholamines and ATP can also leak out of the granule under certain conditions (see earlier). For a catecholamine to ATP molar ratio of 4 to 1 the charge on catecholamines is balanced by that on the nucleotides and,therefore, some kind of complex involving these two compounds was anticipated.

In vitro studies, however, have shown only a weak interaction between adrenaline and ATP (Pai and Maynert, 1972; Weiner and Jardetzky, 1964) even in concentrated solution (Berneis et al., 1969). The addition of a divident metal ion (usually Mg^{++} or Ca^{++}) to the catecholamine and ATP results in a second more dense liquid phase separating out (Berneis et al., 1970). The ability to form this high molecular weight complex is dependent on the catecholamine present, the temperature and calcium content. Unfortunately, at 37°C and in the absence of other stabilizing factors much higher concentrations of calcium than are actually found in the granule matrix are required for complex formation.

Chromogranins do not possess any high affinity binding sites for catecholamines as shown by equilibrium dialysis experiments (Smith and Kirshner, 1967) but chromogranin A can form a gel at high concentrations in vitro (Smith, 1968; Strieder et al., 1968). The presence of chromogranins tends to prevent the formation of the catecholamine:ATP:Ca^{++} referred to above. However, it may be that the negative charge on the chromogranins or its possible gel structure in concentrated solution lowers the diffussion rate of the catecholamines.

A gel-like structure for the matrix is not supported by NMR

(Daniels et al., 1974; Sharp and Richards, 1977a,b; Caterall et al., 1973) and fluorescence (Steffen et al., 1974) studies which indicate that the internal environment of the granule is no more viscous than water. Furthermore granules flatten when dehydrated (Morris, see Apps and Phillips, 1979) ruling out a non-compressible matrix. However, it is possible that at $0^{o}C$ complexes involving catecholamine, ATP and Ca^{++} may form in isolated granules. NMR studies reveal the following: the chromogranins are in a random coil configuration and are freely tumbling; ATP and catecholamines are in different environments and a simple complex between ATP and catecholamines is ruled out. Sharp and Richards (1977b) have, therefore, suggested that ATP cross-links chromogranins through the latter's basic side chains. The osmotic pressure of catecholamines would then be low due to electrostatic interaction with the negatively charged ATP-chromogranin A complex. Fluorescence polarization studies (Steffen et al., 1974) also indicate some sort of interaction between catecholamines and ATP or chromogranins.

Ionophores have also been used to probe the structure of the granule core (Johnson and Scarpa, 1974, 1976b). It would seem that there are high affinity binding sites for calcium in the granule interior and that more than mere electrostatic interaction is required to reduce osmotic pressure since exchange of catecholamine for potassium leads to granule lysis (Johnson and Scarpa, 1976b; Phillips, 1977; Papahadjopoulos et al., 1977).

1.1.14 Catecholamine Uptake by Granules

Because of its specificity, sensitivity to the specific inhibitor reserpine, and ATP dependence, Kirshner (1962) concluded that catecholamine uptake in granules was an active process. Ghosts provide the most favourable experimental system for uptake studies (Phillips, 1974a).

Uptake in ghosts exhibits a number of features indicative of carrier-mediated active transport (see Apps and Phillips, 1979):-

1. Substrate specificity is such that dopamine, adrenaline, noradrenaline, 5-hydroxytryptamine and other phenylethylamine analogues are accumulated (Da Prada et al., 1975; Phillips, 1974b). Lower $S_{0.5}$ values for the natural (-) form of catecholamine than for the (+) form were observed.

2. Uptake is saturable by catecholamines but the initial rate does not show a simple Michaelian dependence on the catecholamine concentration (the Hill coefficient, N_H = 1.2 - 1.3) (Phillips, 1974b).

3. Uptake is ATP dependent although it is not certain whether the translocase requires NTP or whether catecholamine transport is coupled to ion movement down a transmembrane pH gradient or membrane potential produced by a separate ATPase. The ATPase and translocase appear to be distinct having different temperature dependence, pH optima and metal ion requirements.

4. Sensitivity to specific inhibitors, e.g. reserpine, AMP-PCP (Phillips, 1974b) and thiol reagents, such as N-ethylmaleimide (Taugner, 1972, 1976).

5. Production of measurable concentration gradients. 0.13 mM noradrenaline saturates the transport system and produces a concentration of 35 mM noradrenaline in the ghosts assuming an internal volume of 4 µl/mg protein (Phillips and Allison, unpublished).

1.1.15 Involvement of Mg^{++} ATPase in Catecholamine Uptake

The function of the Mg^{++} ATPase of chromaffin granule membranes is the transport of protons from the outside of the granule to the interior. Because of the low passive permeability of the granule membrane

(Johnson and Scarpa,1976b) proton pumping leads to the establishment of a pH gradient and also of a membrane potential since proton movement is electrogenic. The membrane potential will oppose any further proton translocation unless it is neutralized by ion movement e.g. anion uptake

The simplest model for catecholamine uptake produced by pH gradients established across the granule membrane is that the low internal pH causes a 'methylamine type ' distribution. Thus the pH gradient may be abolished by either transport of the neutral form of the catecholamine across the membrane which then becomes protonated in the matrix or by protonated catecholamine inward transport coupled to proton efflux or hydroxyl ion influx. However, the membrane potential still remains. Models combining catecholamine transport with the abolition of both the pH gradient and membrane potential include catecholamine transport and subsequent protonation in the matrix coupled with proton efflux and protonated catecholamine transport coupled to ejection of two protons per protonated catecholamine.

In summary, it is likely that anion uptake is obligatorily coupled to proton movement which generates a large pH gradient which can be used for catecholamine transport. Some proton translocation is probably electrogenic leading to the establishment of a membrane potential which could be used to transport nucleotides.

1.1.16 The Release of Catecholamines

Exocytosis, a calcium dependent fusion of the secretory granule with the plasma membrane resulting in loss of the total content of the granule to the cell exterior through an opening formed at the point of membrane fusion, has been shown to be the mechanism of release of granule

contents in the pancreas, parotid and mast cells (Palade, 1975; Amsterdam et al. 1969; Röhlich et al., 1971). There is overwhelming evidence that exocytosis occurs in the adrenal medulla and this is briefly summarized in the following paragraphs.

Evidence for exocytosis has been obtained by the use of perfusion and biochemical techniques and electron microscopy. Electron micrographs of stimulated cells show the typical omega shaped exocytotic profiles (Grynszpan-Winograd, 1975; Abrahams and Holtzman, 1973). These consist of an invagination of the cell membrane (presumably a granule fused to the plasma membrane) containing what appears to be the electron dense core of a chromaffin granule. These profiles are, however, rarely seen in chromaffin cells and have only been observed in the medullae of the hamster (Grynszpan-Winograd, 1975) and rat (Abrahams and Holtzman, 1973) following stimulation with massive doses of insulin. Freeze-electron microscopy reveals pits on the surface of the cells (Smith et al., 1973) which may be the sites of exocytosis. Furthermore cells stimulated in the presence of horseradish peroxidase contain vesicles in their cytoplasm containing this marker indicating that at one time these vesicles were open to the cell exterior (Abrahams and Holtzman, 1973).

Analysis of the perfusates of stimulated glands revealed the following: the presence of all the soluble components of chromaffin granules (Douglas and Poisner, 1966; Schneider et al., 1967; Schneider, 1969; Dixon et al., 1975) and in the same proportion as they occur in the granule lysate; the cytoplasmic enzyme, phenylethanolamine N-methyl transferase was absent indicating that cytoplasmic contents were not also released; no lipid was present (Schneider et al., 1967) so that it is unlikely that the granule in toto is expelled.

Granules prepared by sucrose density centrifugation from stimulated glands exhibit the same density as granules prepared from unstimulated

glands but the yield is markedly reduced (Viveros et al., 1969). Reserpine treated glands, however, produce the same quantity of granules but of a lesser density (Viveros et al., 1969). Reserpine is known to specifically deplete granules of catecholamines only. Thus exocytosis is seen as an 'all or none' process - if release is to take place then the whole contents of the granule are secreted together.

There is little doubt that, during massive stimulation, and during perfusion experiments with isolated glands an exocytotic mechanism accounts for the secretion of catecholamines by the adrenal medulla. However, it has recently been found that at slower rates of stimulation in the perfused cat adrenal less DβH is released than would be expected from the amount of catecholamine secreted (Dixon et al., 1975). This would seem to indicate a preferential release of small molecules. In nerves the 'all or none' nature of exocytosis also seems doubtful, although in nerves the evidence for exocytosis is less convincing (see Stjärne, 1975 for review) since the quantum of transmitter released is only 10% or less of the amount of transmitter stored in a single vesicle. In some situations the release of just the total contents of one vesicle would lead to a many-fold saturation of the receptor sites. At the neuromuscular junction giant spontaneous potentials are observed and these may be due to the release of the total contents of one synaptic vesicle.

These observations can be explained by the following model suggested by Phillips (Phillips, 1976; Apps and Phillips, 1979). Low levels of stimulation would lead to the elevation of calcium levels for only a brief period time. This would allow some of the granules to make contact with the plasma membrane but rapid removal of calcium by intracellular sinks would prevent actual fusion and full exocytotic release, the granule instead 'dissociating' from the plasma membrane. It is known that

catecholaminescan leak across the granule membrane and it would seem likely that catecholamines can diffuse across the double bilayer in the brief period when the two membranes are opposed to one another.

Thus the key factor governing partial or total release of the granule contents would be the intracellular concentration of calcium at the site of release. The concentration of calcium is presumably controlled by the rate of stimulation which would dictate how long the calcium channels in the plasma membrane remained open and also the affinity of the cell contents for calcium. In chromaffin cells intracellular calcium is removed extremely rapidly and this may explain why exocytotic profiles are only rarely seen and then only after massive stimulation. In exocrine and mast cells the calcium recovery may be slow and this would allow elevated calcium levels to build up deep within the cell and cause the familiar sight of granules fused to each other and the cell membrane.

1.1.17 The Role of Calcium in Exocytosis

The most popular view of the role of calcium is that it brings the two interacting membranes together either by neutralising the charge on the membranes or by cross-linking them (Banks, 1966; Hall and Simon, 1976). It is known that the outer surface of the chromaffin granule is coated with negative charges arising mainly from the carboxyl groups of phosphatidylserine and protein and to a lesser extent from sialic acid (Eagles et al., 1976; Matthews et al., 1972).

Other functions of calcium in exocytosis have been proposed. The direct interaction of calcium with the granule and/or plasma membrane may cause a structural rearrangement of membrane components to take place which would facilitate membrane fusion. Mixed phosphatidylserine liposomes can be fused by the addition of calcium (Papahadjopouloulos et

al., 1977). Chromaffin granules also fuse and rupture in the presence of calcium (Edwards et al., 1974). The attachment regions of these fused granules are devoid of membrane particles as shown by freeze-etching (Schober et al., 1977). The use of spin labels (Marsh et al., 1976) shows that calcium can alter the physical properties of the membrane and it has been hypothesized that calcium may disrupt lysolecithin-cholesterol complexes to leave lysolecithin rich patches (Marsh et al., 1976). Lysolecithin tends to form micelles rather than bilayers and can be used to induce membrane fusion (Lucy, 1970). However, the above events require higher concentrations of calcium than are thought to be required for exocytosis (the concentration of calcium at the plasma membrane during secretion is unknown) and can be mimicked by replacing calcium with magnesium albeit at higher concentrations. Furthermore, other secretory granule membranes do not contain lysolecithin (Da Prada et al., 1972).

The presence of a calcium-activated enzyme could also account for the calcium specificity of exocytosis. Thus there may be a calcium-sensitive phospholipase A_2 which would produce lysolecithin although there is no evidence for such an enzyme in the granule or plasma membrane of the adrenal medullary cell.

1.1.18 Consequences of Exocytosis

A major consequence of exocytosis is the need for membrane retrieval in order to prevent continual expansion of the cell membrane (Palade, 1959). The identification of thorium dioxide and horseradish peroxidase containing vesicles in the cytoplasm of adrenal medullary cells following stimulation in the presence of exogeneous thorium dioxide (Nagasawa and Douglas, 1972) and horseradish peroxidase (Abrahams and Holtzman, 1973), the purification of 'empty chromaffin granules' from heavily

stimulated adrenal glands (Malamed et al., 1968), and the concomitant increase in empty vesicles and decrease in chromaffin granules in the cytoplasm of stimulated medullary cells (Koerker et al., 1973) indicates that membrane retrieval does occur.

The presence of coated pits (Diner, 1967; Grynszpan-Winograd, 1971: Bendeczky and Smith, 1972) seems to indicate which regions of the plasma membrane are to be retrieved. Coated vesicles can be seen in the cytoplasm of cells following stimulation (Nagasawa and Douglas, 1972). Pearse (1975) has isolated a protein, subunit molecular weight 180,000 daltons, which she believes to be involved in coated vesicle formation. The empty vesicles are eventually broken down in lysosomes (Nagasawa and Douglas, 1972; Abrahams and Holtzmann, 1973, Viveros et al., 1969). Phillips (1976) has suggested that under physiological conditions small molecules are preferentially released and these granules which have maintained their soluble proteins should be able to reaccumulate catecholamines and nucleotides.

We can make certain predictions about the arrangement of components of the chromaffin granule membrane in the light of the hypothesis of exocytosis and the known structure of plasma membranes. If we make the not unreasonable assumption that membranes which fuse are likely to be similar in structure and composition (compare, for example, the relatively high cholesterol content of chromaffin granule membranes with plasma membranes) then we would expect the cytoplasmic faces of each membrane to be structually similar and the inner matrix surface of the granule membrane to be equivalent to the outer surface of the cell membrane.

The body of evidence on the organization of the plasma membrane indicates the following structure. Glycoproteins or at least their carbohydrate moieties are exposed on the outer surface of the membrane while

the majority of the protein is present on the cytoplasmic surface of the membrane. There also seems to be an asymmetric distribution of phospholipids in the membrane with sphingomyelin and phosphatidylcholine in the external half of the bilayer of plasma membranes and phosphalidyl-ethanolamine and phosphatidylserine forming the inner half of the bilayer.

Thus we may tentatively suggest that the cytoplasmic surface of chromaffin granule membranes contains most of the protein species of the membrane, all the phosphatidylethanolamine and phosphatidylserine but none of the membrane carbohydrate. In contrast we would expect the surface of the membrane oppossed to the granule matrix to comprise sphin-gomyelin, phosphatidylcholine and all the carbohydrate of the membrane.

1.2 Asymmetric Membrane Labelling Techniques

1.2.1 Introduction

Beside their simple supportive and compartmental roles, biological membranes possess a variety of complex biochemical properties essential to the functioning of cells and organelles. Several of these properties (functions), such as the coupled reactions of the electron transport chain of the inner mitochondrial membrane, coupling of hormone receptors to adenyl cyclase activity in plasma membranes, active transport of metabolites into cells and organelles, and synthesis of proteins into the lumen of the rough endoplasmic reticulum, suggest that membranes must be transversely asymmetrical with respect to structure and composition. Thus the two halves of the bilayer will have different components and the surfaces of the membrane which interact with either the exterior or interior environment of the cell or organelle will be different. This arrangement of components, which presumably is a way of allowing membranes to distinguish between their internal and external environments, is consistent with the studies on the fluidity of membrane components which have shown that the constituents of the membrane are free to diffuse laterally but diffuse only very slowly from one half of the bilayer to the other ("flip-flop"). Thus differences in the composition of the two halves of the bilayer would be maintained.

Labelling studies have been used mainly to recognise which components of the membrane are exposed on the two surfaces of the membrane, particularly the outer surface defined in the intact cell or organelle. Most investigations are concerned with the labelling of proteins and/or glycoproteins; a certain amount of information is also available about the arrangement of lipid in the membrane since lipids are often labelled by the above techniques as well as proteins and glycoproteins. Components

exposed on the outer surface of membranes can be located by labelling intact cells or organelles with a suitable membrane impermeant label (probe), purifying the membrane and separating and analyzing the components of the membrane for the presence of the label. Comparison of the labelling pattern of components when isolated (i.e. open) membranes are used with that from the intact system allows identification of constituents exposed on the inner membrane face. Other methods of recognising components exposed to cell or organelle internal constituents are discussed below.

1.2.2 General Properties Required for Vectorial Labelling

Maddy (1964) has proposed the following set of criteria for reagents used to probe the structure of membranes: firstly they should be membrane impenetrant, secondly they should react at physiological pH, temperature and tonicity, and thirdly they should be detectable in small amounts. Methods of confining labelling to the outer surface of cells or organelles are discussed below but note that the actual label need not be membrane impenetrant. The success of vectorial labelling can be assessed by comparing the incorporation of the label into the membrane fraction with that into the internal contents, such as haemoglobin for erythrocytes (in which case it must be shown that the internal contents do react with the probe when isolated or when the cells or organelles are lysed). Labelling under physiological conditions is a safeguard against lysis or membrane leakiness during the labelling period causing artefactual labelling of internal sites. In erythrocytes the integrity of the membrane during labelling has been monitored by haemoglobin release into the supernatant (Bender et al., 1971), enzyme assays (Shin and Carraway, 1973; Kant and Steck, 1972), and measurement of exclusion or uptake of certain compounds (Triplett and Carraway, 1972; Bodemann, and Passow, 1972).

Detection of the label in small amounts allows greater sensitivity in subsequent analyses as well as the use of a limited low level of labelling in cases where 'over-derivatization' of membranes perturbs the membranes sufficiently to lyse cells or affects the separation properties of the constituents of the membrane thus making analysis difficult.

Other properties of the reagents which are to be desired includes the formation of a covalent bond with the target groups in the membrane. This would ensure that after initial reaction label does not transfer to sites subsequently exposed during or after membrane preparation. Sodium dodecyl sulphate polyacrylamide gel electrophoresis or chromatography in detergent or organic solvents(Triplett et al.,1972a) has been used to establish that label associated with membrane components is covalently linked. Some method of inactivating excess label at the end of the incubation period is also to be desired.

1.2.3 Small Molecular Weight Membrane Impenetrant Reagents

Two classes of membrane impermeable reagents can be defined, viz. those reacting chemically and those enzymatically. Chemical reagents are usually small (i.e. low molecular weight) and in addition to their reactive group possess a charged (hydrophilic) group which prevents them from penetrating membranes. The technique and many of the reagents were originally used to study the structure-function relationships of enzymes, where exposed amino acids were the target groups. Factors influencing labelling include steric accessibility, solvation, general acid or base catalysis, presence of neighbouring charged groups and the membrane acting as a permeability barrier. Some of these factors may increase reactivity compared to individual molecules in solution (Takashi et al., 1967). One of the first reagents to be developed for vectorial

labelling was 4'-isothiocyanostilbene-2,2'-disulphonic acid (SITS), a fluorescent compound reacting with primary amino groups (Maddy, 1964).

Berg (1969) has used the diazonium salt of sulphanilic acid to label amino and sulphydryl groups as well as tyrosine and histidine residues of proteins exposed on the outside of erythrocytes. Other small molecular weight impenetrant reagents are listed in Table 1.2. Membrane permeant labels, e.g. N-ethylmaleimide (Carraway and Shin, 1972) and acetic anhydride (Carraway et al., 1972), can be combined with impenetrant reagents to show the latent reactivity of membrane components which are not labelled in intact cells by impenetrant reagents. Isolated membranes or leaky ghosts reacted with impenetrant probes can also be used to show which components are on the inner surface of the membrane. (however, see below).

In practice most chemical 'impenetrant'reagents have a finite membrane permeability due to equilibrium between ionised and unionised forms(the ionised form being impermeable but the unionised form having some degree of penetrability) and/or delocalisation of the charge. This problem is exemplified by experiments which have shown that some penetrant and impenetrant reagents give identical labelling patterns of membrane components when intact erythrocytes are used (Schmidt-Ullrich et al., 1973). The penetrant reagents labelled haemoglobin when intact erythrocytes were reacted. Carraway (1975) has produced a simplified scheme of interactions between the membrane and the impenetrant reagent (see Fig. 1.2). True vectorial labelling occurs if the rate of reaction of the probe with groups exposed on the outside of the membrane (k_x) is much greater than its rate of penetration across the membrane (k_1). This can be 'checked' by measuring incorporation into haemoglobin, in the case of erythrocytes. However it has been suggested that the presence

Reagent name	Abbreviations	Target gp.	Refs
Isothiocyanostilbene disulphonic acids	SITS	NH_2	1,2,3
	DIDS		4,5,6
Diazotized sulphanilic acid	DSA	NH_2,SH	7,8,9
		tyr,his	10
Formylmethionylmethyl phosphate	FMMP	NH_2	11,12,
			13,14
Pyridoxal phosphate-borohydride		NH_2	6,15,16
Trinitrobenzene sulphonate	TNBS	NH_2	17,18,19
			20,21
N-(4-azido-2-nitrophenyl)	NAP-taurine	Non-specific	22,23,24
2-aminoethane sulphonate			
Fluorescamine		NH_2	25,26,27
Isethionyl acetimidate	IAI	NH_2	28

Table 1.2 Small Molecular Weight Impenetrant Probes Used for Vectorial Labelling of Membrane Proteins

Modified from Carraway (1975)

References: 1. Maddy (1964); 2. Cabantchik & Rothstein (1974b); 3. Cabantchik & Rothstein (1972); 4. Cabantchik & Rothstein (1974a); 5. Juliano (1974); 6. Juliano & Behar-Bannelier (1975); 7. Carraway et al. (1971); 8. Berg (1969); 9. Tinberg et al. (1974); 10 Konig et al. (1976); 11. Bretscher (1972); 12. Bretscher (1971a); 13. Bretscher (1971b); 14. Bretscher (1971c); 15. Rifkin et al. (1972); 16. Hunt & Brown (1974); 17 Arrotti & Garvin (1972a); 18. Gordesky & Marinetti (1973); 19. Vidal et al.(1974); 20. Arrotti & Garvin (1972b);21. Tarone et al. (1973); 22. Staros et al. (1974); 23. Staros & Richards (1974); 24. Richards et al. (1975); 25. Hawkes et al. (1976); 26. Nakaya et al. (1975); 27. Hildalgo & Ikemoto (1977); 28. Whiteley & Berg (1974).

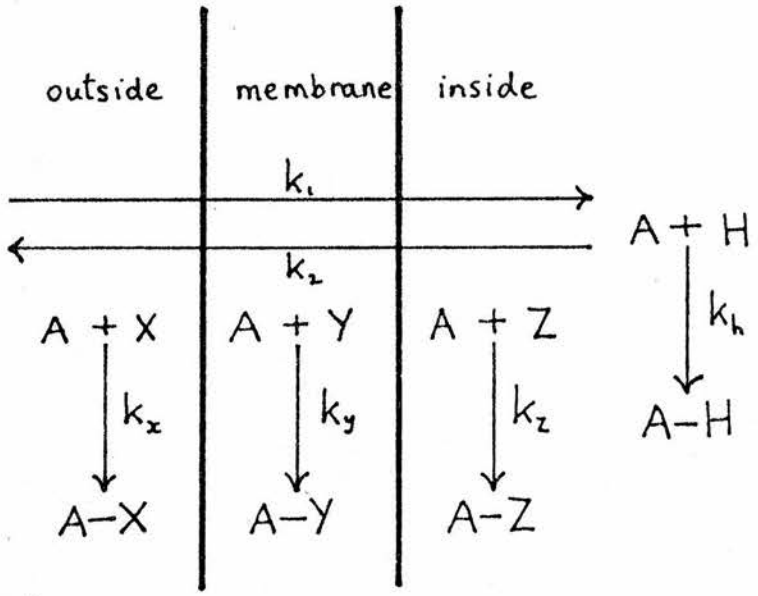


Fig. 1.2 Model For The Evaluation of The Reactivity and Permeation of Membrane Reagents

Reproduced from Carraway (1975). For details see text.

of a high concentration of reactive groups in the cell or organelle matrix could act as a sink, 'mopping' up reagent that has penetrated the membrane and preventing reaction at the inner surface of the membrane (Arrotti, and Garvin, 1972). Therefore vectorial labelling could also occur if the concentration of matrix proteins ($[H]$) was large and the rate or reactivity of 'matrix' proteins (k_h) high compared to the concentration of reactive components on the inner surface of the membrane ($[Z]$) and their rate of reacting with the probe (k_z). However, specific interactions such as those occurring with iodoacetate (Carraway and Shin, 1972) and membrane binding of the reagent which may be covalent or otherwise may interfere with the above.

Permeant labels can also be used to determine which reactive groups are localized at one surface or the other. p-chloromercuribenzoate (PCMB) freely traverses the erythrocyte membrane to react with exposed sulphydryl groups on both membrane faces. However, labelling with PCMB can be completely removed by glutathione and because this compound is non-penetrating it will only dissociate PCMB from the outer surface of the membrane of PCMB reacted erythrocytes.

Staros and Richards (1974) have introduced the technique of photochemical labelling which involves the use of the compound N-(4-azido-2-nitrophenyl)-2-aminoethane sulphonate. Light converts this reagent to a highly reactive nitrene derivative which can insert into C-H bonds to form arylamine derivatives. Erythrocytes can also be loaded with this compound, excess reagent removed from the exterior by washing and photolysis carried out at $0^{o}C$ resulting in labelling of the inner surface of the membrane and haemoglobin (Richards et al., 1975). The high reactivity of this compound coupled with the widespread occurrence of the target group should enable this reagent to label every component exposed at a particular surface. The reagent is rapidly inactivated

by water. However, protein polymerization may occur which could confuse analysis of labelling (Richards et al., 1975; Ruoho and Kyte, 1974).

A more sophisticated approach to vectorial labelling with chemical reagents involves the use of large molecular weight carbohydrate chains as 'carriers' for the labelling reagent, the two being covalently linked. Himmelspach and associates (1971) have used flavozoles covalently linked to isomaltose (up to eight residues) to analyze proteins on the membranes of intact erythrocytes, erythrocyte ghosts as well as normally oriented and inverted membrane vesicles. Use of these types of reagents allows greater versatility in studying membrane structure since their size can be controlled (e.g. by using between 1 to 8 isomaltose units above); the carbohydrate can be removed either by periodate cleavage between the vicinal glycols or enzymatically, adding another element of spatial discrimination; the carbohydrate groups are antigenic; providing additional means of detection and localization, the carbohydrate chains can be used as carriers for amino and sulphydryl reagents as well as photoaffinity labels and protective groups; bifunctional reagents with the reactive groups separated by the carbohydrate chains (whose length can be varied) can be used to analyze protein distribution within the plane of the membrane.

1.2.4 Enzymic Labelling

The chief advantage of enzymic labelling over chemical labelling (apart from reagents derivatized with large molecular weight carbohydrate residues) is that the enzyme cannot penetrate membranes to any extent (provided active transport of proteins or lysis or leakiness of intact membrane systems does not occur), because of their large size. However, one must perform controls to show that any labelling is enzyme dependent and that in the absence of the enzyme labelling of the components is negligible.

The most common enzyme used to study exposed proteins on the surface of membranes is lactoperoxidase. In the presence of iodide and hydrogen peroxide this enzyme iodinates accessible tyrosine and possibly histidine residues of proteins (Marchalonis, 1969). Reaction occurs at neutral pH, low iodide concentration and via an enzyme bound intermediate in which iodide is transferred directly to protein (Morrison et al., 1971). The level of hydrogen peroxide must be kept low to prevent lipid oxidation. This can be achieved by successive addition of small amounts of hydrogen peroxide (Phillips and Morrison,1971a) after fixed intervals or by the use of a glucose/glucose oxidase hydrogen peroxide generating system continually supplying a low concentration of hydrogen peroxide (Hubbard and Cohn, 1972). Labelled proteins can be detected by radioactive iodine, either ^{125}I or ^{131}I (see below).

The 'lactoperoxidase labelling technique' has become a routine procedure for studing exposed proteins in membranes and has been used to study the structure of the membranes of erythrocytes, lymphocytes, viruses, platelets, and slime moulds as well as the membranes of organelles (the inner membrane of mitochondria, the sarcoplasmic reticulum and rat liver rough microsomes).The versatility of this method has been demonstrated in studies with the erythrocyte membrane. Not only have intact erythrocytes been labelled (Phillips and Morrison, 1970,1971a) but also 'leaky' ghosts (Marchesi et al.,1972) and 'inside-out' resealed ghosts (Reichstein and Blostein 1975). Furthermore, lactoperoxidase can be sealed into membrane vesicles (Shin and Carraway 1974; Reichstein and Blostein 1973) so that proteins exposed on the inner membrane face can be labelled since iodide and hyrogen peroxide are free to permeate membranes. Mueller and Morrison (1974) added catalase to resealed ghosts containing lactoperoxidase in order to

prevent disgorged lactoperoxidase from labelling the outside of the vesicles. Catalase will compete with lactoperoxidase for hydrogen peroxide. Intact erythrocytes can be iodinated by lactoperoxidase with one radioisotope of iodine, leaky ghosts prepared and then iodinated by lactoperoxidase with the other radioisotope of iodine. Transmembrane proteins can then be recognised as containing both radioisotopes of iodine (however, see later).

Chief problems which may exist when using lactoperoxidase (see Morrison, 1974) include labelling of itself (and any other proteins which may be present in the enzyme preparation). If membranes or cells are not sufficiently purified iodinated lactoperoxidase may appear in the labelling profile and be mistaken for a labelled membrane protein. The use of low enzyme concentrations to reduce self-labelling may result in enzyme inhibition. Furthermore lipid labelling may be indicative of the generation of I_2 which is free to cross membranes. Hence controls such as iodination in the absence of lactoperoxidase or analysis of matrix proteins for radioactive iodine should always be performed.

Glycoproteins (and glycolipids) can be labelled by the galactose oxidase/$[^3H]NaBH_4$ technique. The 6 position of galactose or N-acetylgalactosamine residues is oxidized by galactose oxidase to an aldehyde group which can be reduced with tritiated borohydride during (Steck and Dawson, 1974) or after (Gahmberg and Hakomori, 1973a) oxidation. This method can also be coupled with prior neuraminidase treatment which usually increases the amount of labelling. Carbohydrate is an important component of the outer surfaces of cell membranes and the effect of transformation, the state of growth or the stage of the cell cycle on the pattern of labelling of intact cells with galactose oxidase/$[^3H]BH_4$ has been followed.

A related approach to enzymatic labelling involves proteolytic digestion with various proteases (see Wallach, 1972, for review). Here, exposed proteins are degraded rather than labelled. Proteases have been used to confirm localization of certain antigens at the external surface of plasma membranes. However, this approach has allowed isolation and characterization of peptide molecules bearing certain blood group haptens, histo compatibility specificities, and other antigenic determinants. Proteolytic digestion can also uncover "cryptic sites" not normally accessible to the reagents which interact with these sites. These cryptic sites may also become accessible upon neoplastic transformation. The structure of nerves has also been probed using a variety of different proteases each attacking different peptide bonds. A major break-through in the use of proteases to study membrane structure was the utilisation of sodium dodecyl sulphate polyacrylamide gel electrophoresis to separate the membrane proteins (see below). Because this method separates proteins according to their molecular weight digested proteins are altered in mobility. Comparison of the polypeptide profile of untreated membranes with that of digested membranes allows susceptible proteins to be identified easily. Furthermore, digested cells or membranes can be labelled with either chemical or enzymatic membrane impenetrant probes since "cryptic sites" will be exposed. This method of investigation and problems associated with it are more fully discussed in Chapter 5.

1.2.5 Determination of Membrane Labelling Pattern

The generally accepted method of analysing the protein and glcyoprotein components of membranes is by sodium dodecyl sulphate polyacrylamide gel electrophoresis (Lenard, 1970). Column chromatography in detergent (Rosenberg and Guidotti, 1969) and isoelectric focussing

(Bhakdi et al., 1974) have also been used but they lack the resolution, simplicity and versatility of SDS gel electrophoresis. Recent advances, such as the use of 'slab' gels as opposed to disc gels which allows several samples to be run side by side under identical conditions (Ames, 1974) and discontinuous buffer systems (Neville, 1971) have enhanced the resolving powers of electrophoresis even further. This technique is discussed in detail elsewhere but a few points salient to membrane labelling will be mentioned here. SDS polyacrylamide gel electrophoresis separates polypeptides according to their molecular weight but for a variety of reasons, particularly abnormal detergent binding, molecular weights calculated by this technique may be incorrect especially for glycoproteins (Segrest et al., 1971; Grefrath and Reynolds, 1974). Any band detected by a particular stain may represent more than one polypeptide species. Heavy modification with a particular probe may alter SDS binding and hence the electrophoretic mobility of a polypeptide. Proteins can be detected in gels after electrophoresis by staining with coomassie brilliant blue. This stain can be used to estimate the amount of a particular polypeptide since staining is usually proportional to amount (Fairbanks et al., 1971) but its proportion in a mixture of polypeptides cannot be calculated by comparison of staining intensities since different polypeptides may bind different amounts of dye per unit weight. Carraway et al. (1971) have shown that heavily glycosylated proteins stain very poorly or not at all with CBB. Dye binding may be altered by labelling e.g. because of the introduction of a high negative charge density. Glycoproteins can be separately visualised with the periodic acid - Schiff (PAS) stain. This stain requires prior removal of SDS from the gels to be stained (Glossman and Neville, 1971), detects mainly sialoglycoproteins (Liao,et al., 1973).

and may not detect glycoproteins containing relatively little carbohydrate and no sialic acid (Tanner and Boxer, 1972). Quantitation of a particular glycoprotein by the PAS stain is also suspect.

It has been shown that SDS polyacrylamide gel electrophoresis fails to separate some membrane proteins or to convert them to the rod shape that most proteins are assumed to adopt during SDS gel electrophoresis (Reynolds and Tanford, 1970a). It has also been reported that two glycoproteins identified by this procedure, from the human erythrocyte membrane, exhibit a monomer-dimer relationship (Marton and Garvin, 1973; Tuech and Morrison, 1974).

Most probes label polypeptides radioactively, fluorescently or chromophorically and methods exist for detecting proteins derivatized in this manner in acrylamide gels. The simplest method of locating radioactivity in acrylamide gels is by autoradiography. This is convenient for ^{32}P, ^{35}S, ^{125}I, ^{131}I and the wet gels can be wrapped in cellophane and placed in contact with X-ray film. Better resolution and shorter exposure times are achieved by drying down the gels before autoradiography. ^{3}H and ^{14}C cannot be detected by conventional autoradiography and a special technique known as fluorography has been developed (Bonner and Laskey, 1974). Gels are impregnated with fluor and exposed to X-ray film at $-70^{o}C$ in the frozen or dried down state. After development X-ray films can be densitometrically scanned and compared to a scan of the polypeptides or glycoproteins of the membrane. Alternatively radioactivity can be directly detected in gel slices. ^{125}I can be counted by gamma spectrometry. For ^{3}H and ^{14}C the gel slices can be 'solubilized' with a suitable base such as hyamine or oxidized with hydrogen peroxide or treated with both, fluor is added and the mixture counted in a liquid scintillation spectrometer. With the slicing technique minor radioactive bands may not be detected since

many bands will be much smaller than the width of the gel slice. Care must also be taken to treat each gel slice identically. Acrylamide gels in which radioactivity is to be determined can usually be stained prior to this determinsation.

Fluorescence is usually more difficult to detect requiring sophisticated instrumentation. Gels can be sliced and fluorescence in each slice quantitated by fluorimetry. Alternatively intact gels can be scanned for fluorescence using either a suitably modified linear tranport spectrophotometer or fluorescence microscope equipped with a motorized stage, both connected to chart recorders. After fluorescence scanning gels can be stained and scanned densitometrically.

Chromophorically labelled proteins separated by SDS polyacrylamide gel electrophoresis can be detected immediately after electrophoresis by densitometric scanning in a spectrophotometer equipped with a linear transport facility. SDS polyacrylamide gel electrophoresis also confirms the covalent nature of the linkage between the label and polypeptide. Chromatography in the presence of detergent or organic solvents (Triplett et al., 1972a) can also be used to this end.

1.2.6 Interpretation of Results of Vectorial Labelling Studies

In interpreting the results of labelling experiments one must be aware of several general problems. Absence of label associated with polypeptides when intact cells or organelles are labelled by an impenetrant technique does not necessarily indicate that this particular polypeptide species is missing from the outer surface of the membrane. As discussed earlier a variety of factors influence labelling beside the presence of a permeability barrier. When isolated membranes are labelled the appearance of label associated with a protein not labelled when intact cells or organelles are used is usually interpreted to

indicate that this protein is associated with the inner face of the membrane. However, it has been suggested that when cells or organelles are lysed the membrane as a whole or individual proteins may undergo conformational changes exposing new sites or proteins at the outer surface of the membrane (see Carraway, 1975). These changes may also result in the artefactual exposure of proteins at the inner surface of the membrane. It is well known that resealed erythrocyte membrane ghosts are deficient in certain proteins as well as differing in certain permeability and ion transport functions. The concept of structural changes during membrane preparation is important in studying "transmembrane" proteins i.e. proteins that span the lipid bilayer and are exposed at both membrane surfaces. It is not sufficient to show that a protein can be labelled with an impenetrant probe in isolated membranes and intact cells or organelles or in inside-out vesicles or by sealing reagent inside right-side out orientated vesicles. Whiteley and Berg (1974) have used related non penetrating (isethionyl acetimidate) and penetrating (ethyl acetimidate) reagents in order to locate proteins spanning the erythrocyte membrane. Intact cells could then be reacted with ^{14}C labelled isethionyl acetimidate or ^{3}H ethyl acetimidate. Pronase was used to locate labelled peptides at the surface of the membrane. They found that components III and IVa probably span the membrane. Studies of this kind where proteins exposed on the inner membrane face are labelled in intact cells or organelles rule out the posssible labelling of artefactually exposed reactive sites. However the possibility that a particular protein is present on both sides of the membrane but individual protein molecules are only exposed at one surface and do not span the membrane has not been ruled out. This would necessitate proof of both types of label present in indivi-

dual protein molecules when intact cells or organelles are first labelled with ^{14}C isethionyl acetimidate under saturating conditions and then with ^{3}H ethyl acetimidate.

In surface labelling of complex cells there has been a tendency for whole cells to be subjected to SDS polyacrylamide gel electrophoresis, followed by analysis for the label. "Complex" cells are generally less stable than erythrocytes. Dead cells could become labelled internally. Thus the label associated with cytoplasmic proteins or proteins exposed on the inside surface of the cell membrane would be included in the profile of proteins exposed on the outside of the cell membrane. When analyzing the surface labelling of complex cells it seems desirable to purify the plasma membrane and show that the label purifies with it. Plasma membrane should then be subjected to electrophoresis in the presence of SDS and analyzed for label. Controls performed should include analysis of cytoplasmic contents for label when intact cells are labelled, isolated membranes should also be labelled and, in the case of enzymatic labelling the reaction should be shown to be enzyme dependent.

1.3 STRATEGY FOR ASYMMETRIC LABELLING OF THE CHROMAFFIN GRANULE MEMBRANE

The topography of the membrane proteins of the chromaffin granule has been determined by lactoperoxidase catalyzed radioiodination, pronase digestion and reaction with N-(iodoacetylaminoethyl)-5-napthylamine-1-sulphonic acid, a fluorescent reagent for cysteine residues. In addition the carbohydrate moieties of the membrane glycoproteins have been localized by tritiation with [^{3}H]-borohydride following galactose oxidase or periodate treatment.

Chromaffin granules become increasingly fragile as they are purified and therefore a relatively crude preparation of granules heavily contaminated with other organelles was used for the various labelling experiments. Intact granules were subsequently purified from contaminating organelles and also damaged granules which may have been labelled at both membrane surfaces. After membrane preparation the polypeptides were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and analyzed for the presence of label. The permeability of the various labels was assessed by measuring the labelling of the intra-granular proteins prepared from labelled intact granules. Isolated membranes were also labelled by the various techniques for comparison with membranes prepared from the labelling of intact granules.

CHAPTER 2

GENERAL METHODS

GENERAL METHODS

2.1 Subcellular Fractionation

2.1.1 Preparation of P_2 or Large Granule Fraction

The method of Smith and Winkler (1967a) was followed. Adrenal medullae were carefully dissected from bovine adrenal glands (which had been kept in ice during transport from a local slaughterhouse to the laboratory), placed in ice cold 0.3 M sucrose bufferred with 10 mM hepes at pH 7, minced with a tissue mincer, and homogenised using a motor-driven teflon homogeniser. Unbroken cells, cell nuclei etc. were removed by centrifugation at 550 g-av for 10 minutes (Beckman low speed centrifuge, JA-20 rotor, 2°C) and the supernatant respun at 18,000 g-av for 15 mins. at 2°C. The pellet was washed with 0.3 M sucrose, 10 mM hepes, pH 7, resuspended in the same buffer and recentrifuged at 18,000 g-av. The second pellet was also washed with 0.3 M sucrose, 10 mM hepes, pH 7 and resuspended in this buffer to yield the P_2 or large granule fraction.

2.1.2 Preparation of Chromaffin Granule Membranes and Lysate

Chromaffin granules were further purified by sedimentation of the P_2 or large granule fraction through 1.8 M sucrose (Schneider, 1972). 5 ml P_2 granules were layered above 20 mls 1.8 M sucrose, 10 mM hepes, pH 7 and centrifuged at 120,000 g-av for 1 hr at 2°C in a Beckman L2 65B ultracentrifuge using a Type 42.1 rotor. The 0.3 M and 1.8 M sucrose layers were carefully removed by aspiration and the pellet resuspended in a minimal volume of 1.8 M sucrose, 10 mM hepes, pH 7 before dilution with a 40 - fold volume of 10 mM hepes, pH 7 in order to lyse the granules by hypotonic shock. Freeze-thawing was not employed (Winkler et al., 1970). The lysed granules were subjected to centrifugation at 160,000 g-av

for 1 hr at 2°C (Beckman L2 65B,Type 65 rotor). The supernatant was retained and represents the preparation referred to, hereafter, as the chromaffin granule lysate and was purified no further unless specified. The pellet was resuspended in a small volume of 1.8 M sucrose, 10 mM hepes, pH 7., subjected to hypotonic shock by rapid dilution with 10 mM hepes, pH 7, and recentrifuged at 160,000 g-av for 1 hr. The resuspension, hypotonic shocking and centrifugation procedures were repeated for a third time and the pellet, mainly comprising washed chromaffin granule membranes, was resuspended in a small volume of 10 mM hepes, pH 7 to approximately 5 - 10 mgs protein/ml.

2.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

2.2.1 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis in 8% and 10% (w/v) Acrylamide Gels

Samples were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis in 15 cm by 14 cm by 1.5 mm thick 8% or 10% (w/v) acrylamide gels containing 0.213% and 0.267% (w/v) NN'methylenebisacrylamide respectively according to the method of Laemmli (1970) and using the slab gel electrophoresis apparatus produced by Raven Scientific Limited. The gels were polymerized with ammonium persulphate (0.75 mg/ml) and by the addition of 0.025 ml per 30 mls separating gel tetraethylmethylenediamine (TEMED). Secondary butanol saturated with water was used to create a flat surface to the top of the gel and was removed after polymerization was complete which normally took 15 - 20 mins. A 3% acrylamide, 0.08% methylenebisacrylamide stacking gel, 2 cms deep, was employed. Sample wells,0.85 cm wide by 1.6 cm deep, were formed in the top of the stacking gel by the insertion of a perspex comb prior to polymerization which was performed in analogous manner to that used for the separating gel.

Samples were prepared for electrophoresis by the addition of an equal volume of a sample buffer consisting of 0.12 M tris-HCℓ (pH 6.8), 10% (w/w) SDS, 80 mM dithiothreitol, 20% (v/v) glycerol and 0.002% (w/v) bromophenol blue and then placed in a boiling water bath for 3 - 4 mins. This treatment was found to be more effective in solubilizing membrane samples than the conditions originally used by Laemmli (1970) as witnessed by the occurrence of less protein at the top of 8% acrylamide gels after gel electrophoresis. Samples to be separated under non reducing conditions of electrophoresis were solubilized as described above except that dithiothreitol was omitted from the sample buffer. After cooling to room temperature the samples were applied to the sample wells with a Hamilton syringe or Drummond microcapillary. Each well could accomodate up to 0.15 mls of sample in sample buffer and thirteen samples could be electrophoresized side by side in the same slab gel.

Electrophoresis was performed at constant voltage, 150 volts being applied to the gel (giving an initial current of 40 mA) to drive the samples through the stacking gel after which the run was completed at 100 volts. The gels were then fixed with 12.5% (w/w) TCA for 1 hr prior to staining with 0.05% coomassie brilliant blue in 5:5:1 methanol:water: acetic acid for 4 hr or overnight or stained directly after electrophoresis. After diffusion destaining with 7.5% acetic acid, 5% methanol, the gels were photographed and, in some cases, individual lanes excised and scanned densitometrically at 570 nm in 7.5% acetic acid, 5% methanol using a Gilford - modified Unicam SP 500 spectrophotometer.

For complex mixtures of proteins, such as membranes, the application of 100 to 150 μgms protein to the gel produced optimum results in terms of resolution and staining intensity. Samples containing relatively few protein species needed relatively less protein to be subjected to electro-

phoresis (of the order of 2 - 3 μgms per individual protein). Reduced and non-reduced samples were not normally separated in the same slab gel but occassionally when they were the two types of sample were spaced apart by at least one empty lane.

Molecular weights of 'unknown' proteins could be calculated from their mobilities (i.e. distance migrated relative to the tracker dye, bromophenol blue) under reducing conditions of SDS polyacrylamide gel electrophoresis using the fact that their mobilities are inversely proportional to the logarithm to the base ten of their molecular weights (Shapiro et al., 1967; Weber and Osborn, 1969). Phosphorylase a (subunit molecular weight 94,000 daltons - see Weber and Osborn, 1969), bovine serum albumin (68,000), catalase (60,000), gamma-globulin, H chain (50,000), ovalbumin (43,000), glyceraldehyde phosphate dehydrogenase (36,000), gamma-globulin, L chain (23,500), soya bean trypsin inhibitor (20,000), and cytochrome c (11,700) were used as reference molecular weight markers and conform to the above relationship (see Fig. 2.1).

2.2.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis in 8% - 20% (w/v) Acrylamide Gradient Gels

8% - 20% (w/v) acrylamide gradient gels, containing 0.375 M Tris-HCℓ (pH 8.8) and 0.1% (w/w) SDS throughout, were prepared using a Watson-Marlowe four channel gradient maker (flow rate 3 - 4 mls/min). The 8% acrylamide solution, in addition, contained 0.213% methylene bisacrylamide, 0.067%TEMED, and 0.7 mgs/ml ammonium persulphate whilst the 20% acrylamide solution contained 0.533% methylene bisacrylamide, 0.067% TEMED, 0.3 mgs/ml ammonium persulphate, and 10% (w/v) sucrose. SDS gel electrophoresis, using a 3% acrylamide stacking gel, was then performed as for 8% and 10% acrylamide gels as described above, except

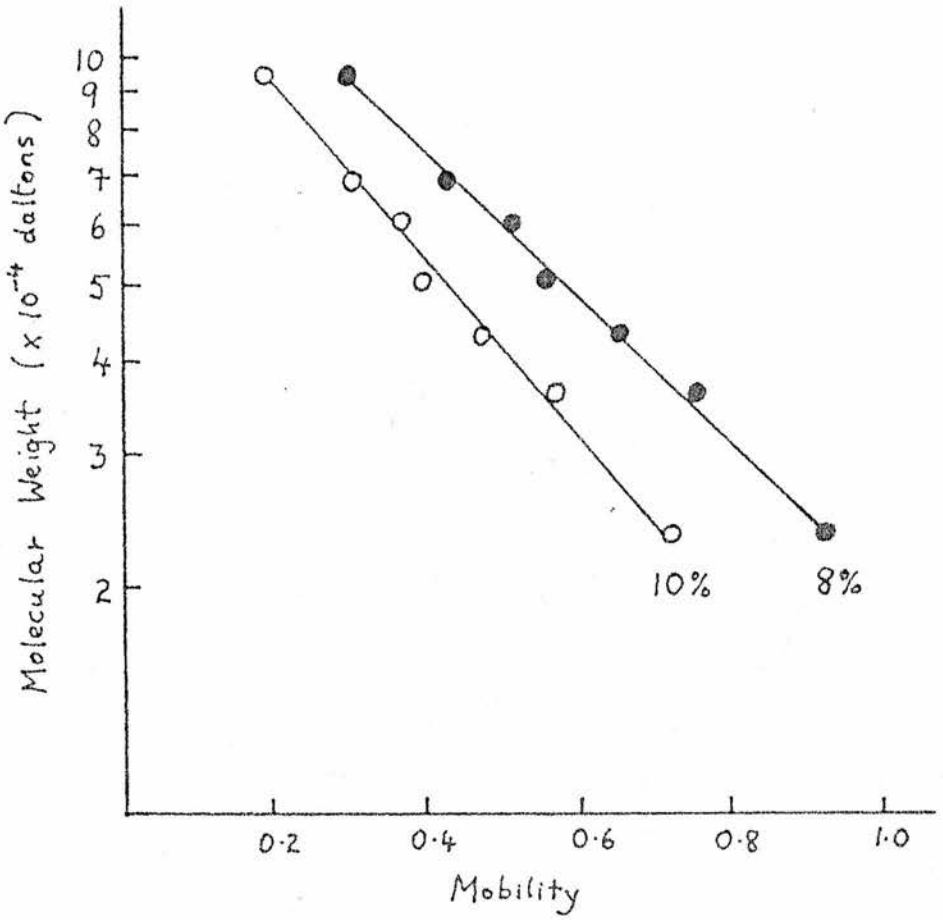


Fig. 2.1 Estimation of Molecular Weight by SDS Polyacrylamide Gel Electrophoresis in 8% and 10% (w/v) Acrylamide Gels.

Approx 5 μgm each phosphorylase a, bovine serum albumen, catalase, H-chain γ globulin, ovalbumen, glyceraldehyde phosphate dehydrogenase, L-chain γ globulin, soya bean trypsin inhibitor and cytochrome c were applied to a single lane of an 8% or 10% (w/v) acrylamide slab gel. SDS polyacrylamide gel electrophoresis was performed under reducing conditions and the gel then stained with coomassie brilliant blue. The mobility of each polypeptide (i.e. the distance migrated relative to the tracker dye, bromophenol blue) was measured from densitometric scans of the gel and plotted against the logarithm to the base ten of the molecular weight of the polypeptide. Cytochrome c migrates with the tracker dye in both 8% and 10% acrylamide gels whilst soya bean trypsin inhibitor also migrates with the tracker dye in 8% acrylamide gels.

that a constant voltage of 40 volts was applied to each gel and the run was performed overnight.

Molecular weights of proteins, separated in polyacrylamide gradient gels in the presence of SDS and a reducing agent, can be determined using the relationship, log (MW) equals a log (T) plus b, where T equals the polyacrylamide concentrated reached by the protein after electrophoresis and a is the slope and b the y intercept of the regression line (Lambin, 1978). It was assumed that the concentration gradient of acrylamide was perfectly linear (which may not, of course, be true). The same reference molecular weight marker proteins used to calibrate 8% and 10% acrylamide gels were employed and conformed to the relationship described by Lambin (1978) when subjected to SDS gel electrophoresis in 8% - 20% polyacrylamide gradient gels (see Fig. 2.2).

2.2.3 Fluorometric Scanning of Polyacrylamide Gels

Individual lanes of polyacrylamide gels, containing fluorescently stained or fluorescently labelled proteins, were placed at an angle of 45^{o} in a quartz boat containing 7.5% acetic acid, 5% methanol and scanned for fluorescence using a Gilford-modified Unicam SP 500 linear transport spectrophotometer (Ragland et al., 1974; Barger et al., 1976). The lid of the spectrophotometer was replaced with a car headlamp bulb (12 V, 24W) and a Corning 7-51 filter used as the excitation filter. The emission filters (Corning 3-72 and 4-96 filters cemented together) and a 0.5 mm vertical slit were interposed between the quartz boat and the photometer. This arrangement of filters prevents reflected incident light from reaching the photometer but allows fluorescence to do so (see Fig. 2.3 for the transmittance properties of the filters at different wavelengths).

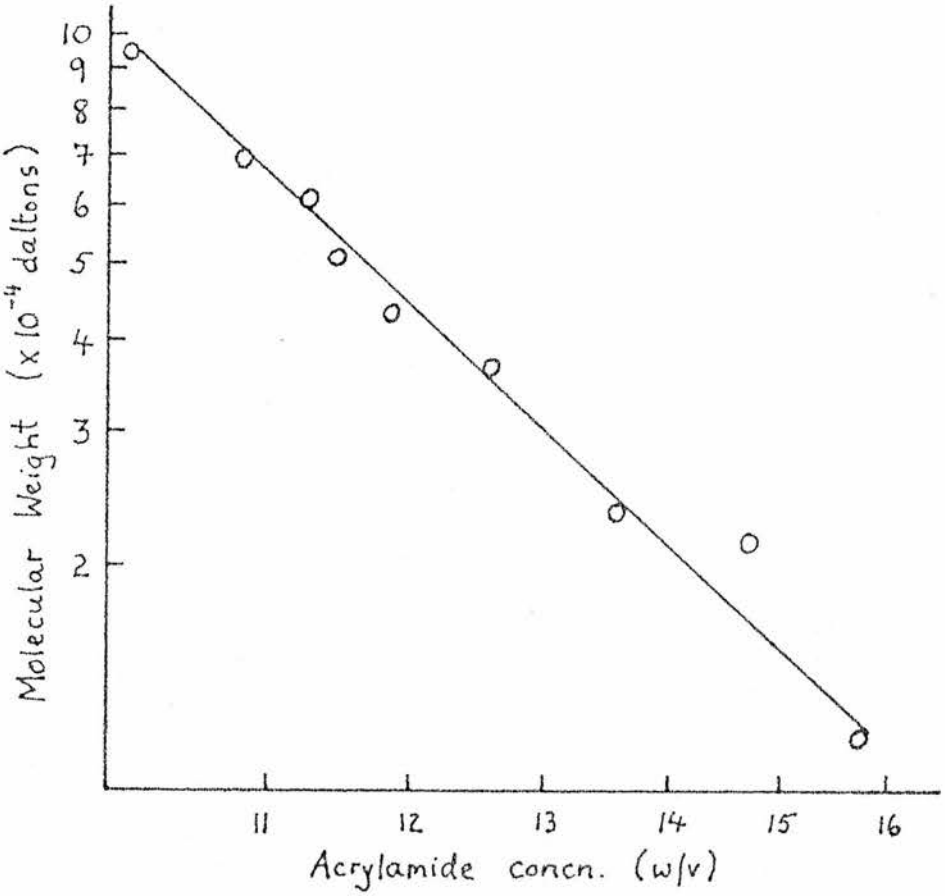


Fig. 2.2 Estimation of Molecular Weight by SDS Polyacrylamide Gel Electrophoresis in 8% - 20% (w/v) Acrylamide Gradient Gels

Various molecular weight markers were separated by SDS polyacrylamide gel electrophoresis in 8% - 20% acrylamide gradient gels under reducing conditions. Approx 5 μgm of each polypeptide was applied to the gel which was stained with coomassie brilliant blue. The logarithm to the base ten of the molecular weight of each polypeptide was then plotted against the logarithm to the base ten of the concentration of acrylamide to which the polypeptide had migrated to in the gradient. It was assumed that the acrylamide gradient was completely linear and the mobilities of each polypeptide (relative to the bottom of the gel) were calculated from densitometric scans of the gel. The molecular weight markers used were phosphorylase a (94,000 daltons), bovine serum albumen (68,000), catalase (60,000), H- chain γ globulin (50,000), ovalbumen (43,000), glyceraldehyde phosphate dehydrogenase (36,000), L-chain γ globulin (23,500), soya bean trypsin inhibitor (20,000) and cytochrome c (11,700)

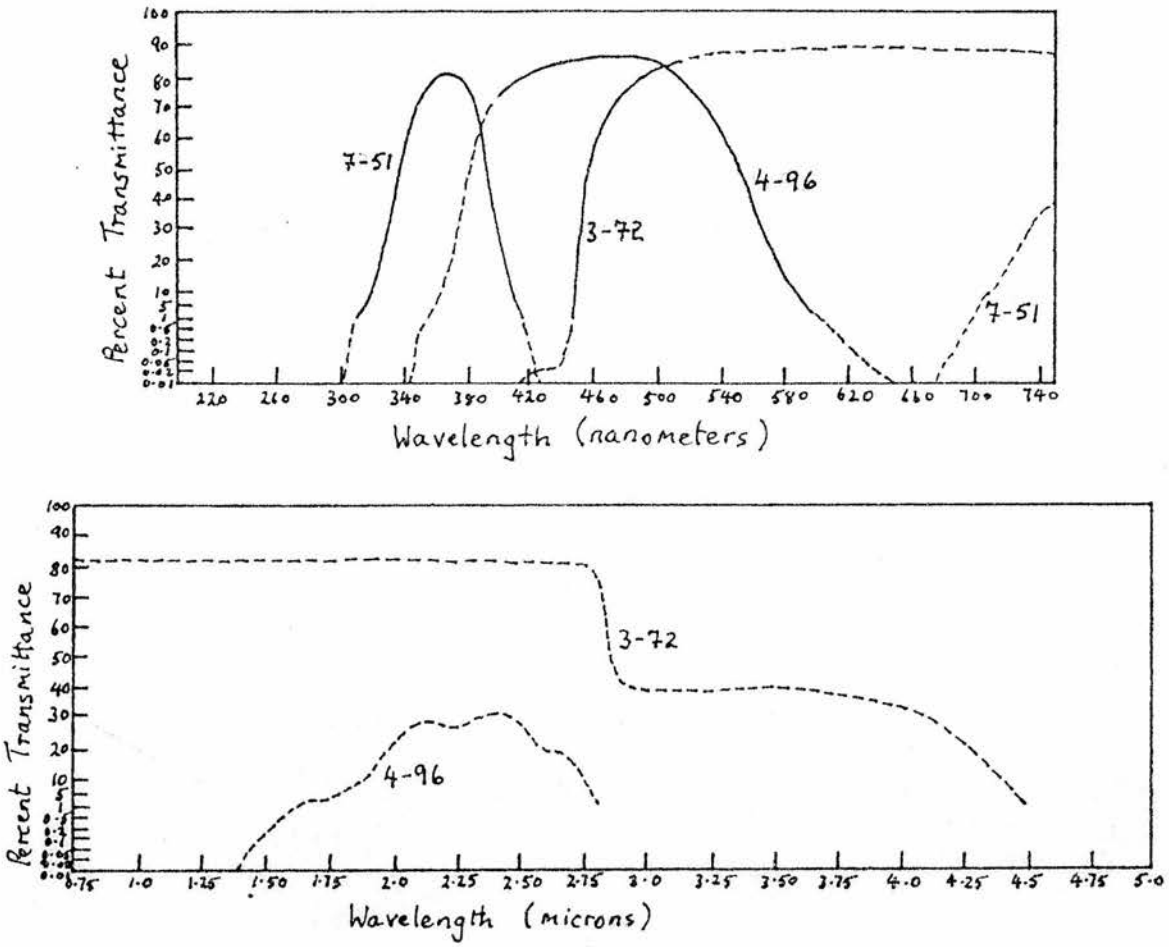


Fig. 2.3 Transmittance Properties of Excitation and Emission Filters Used for Fluorometric Scanning

Fluorometric scanning was compared with densitometric scanning of coomassie brilliant blue stained polyacrylamide gels. Total chromaffin granule lysate (100 µgms protein; see section 2.1.2 for preparation) was subjected to SDS polyacrylamide gel electrophoresis under reducing conditions (see above) in an 8% acrylamide gel. The gel was then stained with anilinonapthalene sulphonic acid (Mg^{++} salt, recrystalized; 0.1 mg/ml in 1% $Na_2S_2O_5$ - 0.1 N HCℓ for 1 - 2 hrs) according to the method of Glossman and Neville (1971) and destained with 1% $Na_2S_2O_5$ - 0.1 N HCℓ until the background was clear when the gel was illuminated with long wavelength ultra-violet light (Chromovue). Fig. 2.4 shows a fluorescence scan of the gel using the modified gel scanner (see above) together with a densitometric scan of the same gel subsequently stained with coomassie brilliant blue in the normal manner (see 2.2.1). The fluorescence scan, which is recorded as negative absorbance on the chart recorder, has been inverted for ease of comparison with the CBB scan. The level of resolution of the fluorescence scan is as good as the densitometric scan although there are differences in the relative heights of the peaks in each scan. This could be due to the fact that the fluorescence signal is linear with percent transmission rather than absorbance.

2.3 Enzyme Assays

2.3.1 Dopamine-β-Hydroxylase

Dopamine-β-hydroxylase (3,4-dihydroxyphenylethylamine β-hydroxylase, E.C.1.14.17.1) activity was estimated essentially as described by Kaufman and Friedman (1965). ^{14}C-tyramine is enzymically converted to octopamine which is chemically converted to p-hydroxybenzaldehyde. The latter can be extracted from the reaction mix with toluene. The enzyme preparation was incubated at 37°C for 30 minutes with 0.22 mM ^{14}C-tyramine (0.1 µCi),

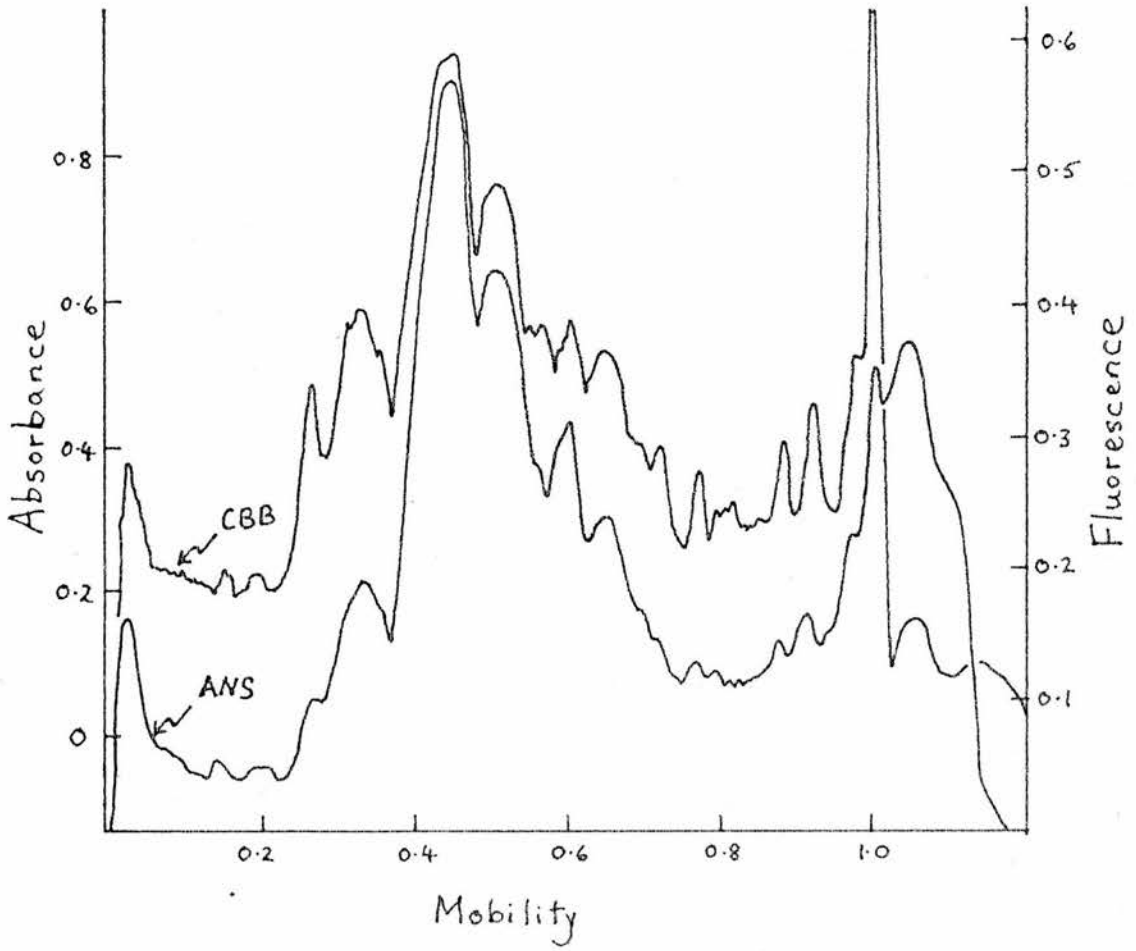


Fig. 2.4 Comparison of Fluorometric Scanning of ANS Stained Gels with Densitometric Scanning of CBB Stained Gels

100 μgm chromaffin granule lysate was subjected to SDS polyacrylamide gel electrophoresis in an 8% (w/v) acrylamide gel under reducing conditions. The gel was then stained with anilinonapthalene sulphonic acid according to Glossman and Neville (1971) and fluorometrically scanned using the modified linear transport Gilford spectrophotometer. The same gel was subsequently stained with coomassie brilliant blue and densitometrically scanned at 570 nm. The fluorescence scan has been inverted for ease of comparison.

0.1% triton X-100, 1% ethanol, 1 mg/ml bovine serum albumin, 0.5 M ascorbate, 0.25 mg/ml catalase (13,300 sigma units/mg), 0.001 mM p-hydroxymercuribenzoate (to inhibit endogeneous DβH inhibitors), 5 mM iproniazid phosphate (a monoamine oxidase inhibitor), and 50 mM fumarate-NaOH (pH 6.5) in 100 mM potassium phosphate buffer, pH 6.5. The reaction was stopped with 0.1 ml 20% (w/w) TCA (ice cold). After equilibration to room temperature 50 µl concentrated ammonia and then 25 µl 2% (w/v) $NaIO_4$ were added. Four minutes later 0.3 ml 8% (v/v) glycerol/3.7 N HCℓ and then 0.5 ml toluene were also added. The mixture was vortex mixed for 30 seconds and spun briefly in the bench centrifuge. 0.25 ml of the toluene layer was extracted and counted by liquid scintillation spectrometry using toluene fluor. Enzyme blanks were also 'assayed' to assess the solubility of tyramine in toluene.

2.3.2 Monoamine Oxidase

The method used to assay monoamine oxidase (E.C.1.4.3.4) activity is based upon that described by Wurtman and Axelrod (1963). To the enzyme preparation was added 0.1 ml 0.0227 mM ^{14}C-labelled tyramine (0.1 µCi total), 0.1 M hepes, pH 7.2. After incubation for 15 min. at 37°C the reaction was stopped with 0.1 ml 2 N HCℓ and the p-hydroxyphenylacetaldehyde formed extracted with 0.25 ml toluene by vortex mixing for 15 seconds and briefly spinning in the bench centrifuge. 0.15 ml of the toluene layer was removed and counted by liquid scintillation spectrometry using toluene fluor. Again, enzyme blanks were carried through the procedure to assess carry-over of ^{14}C-tyramine into the toluene layer.

2.3.3 NADH:Acceptor Oxidoreductase

The method of Flatmark et al. (1971) was adopted in order to measure the NADH:acceptor oxidoreductase activity (E.C.1.6.99.3) of chromaffin granule membranes. Membranes were added to a quartz cuvette con-

taining 50 mM hepes, pH 7, 0,1 mM potassium ferricyanide and 0.025 mM NADH at room temperature and the change in absorbance at 340 nm followed on a chart recorder linked to the spectrophotometer over a 10 minute period. Enzyme activities were calculated from the initial slopes of the chart recordings. (The non enzymic reduction of the acceptor by NADH was ignored).

2.3.4 Mg^{++} activated ATPase (E.C.3.6.1.4)

Muszbek et al.'s(1977) highly sensitive method for the measurement of ATPase activity was used. To the enzyme preparation was added 0.1 ml 1 mM $MgC\ell_2$, 1 mM ATP, 10 mM hepes, pH 7 and the whole incubated at 37°C for 30 mins. The reaction was stopped by the addition of 1.5 ml 5% (w/w) TCA (ice-cold) and the amount of phosphate released measured as described by Muszbeck et al. (1977) using 1 mM potassium phosphate as standard. Thus, by this procedure 10 µgms protein of chromaffin granule membranes yielded an absorbance of between 0.4 and 0.5 absorbance units. For each sample control values, obtained by the addition of ice-cold 5% TCA to the sample immediately after the incubation medium had been added and then phosphate measurement, were subtracted.

2.3.5 Phosphatidylinositol Kinase (E.C.2.7.1.67)

The phosphatidylinositol kinase activity of chromaffin granule membranes was assayed by the incorporation of ^{32}P into the membranes from γ-^{32}P labelled ATP according to Phillips (1973). Controls (incubations in the presence of 2 mM EDTA instead of the $MgC\ell_2$ and $MnC\ell_2$) were subtracted from the test values to give the enzyme dependent incorporation of ^{32}P from γ-^{32}P labelled ATP into the membranes.

2.3.6 Cytochrome b-561

The reduced versus oxidized difference spectrum of chromaffin granule membranes in 10 mM hepes, pH 7 was scanned in a dual beam

spectrophotometer.

Samples were reduced with dithionite and untreated samples were assumed to be fully oxidized. The protein concentration was 0.05 mg/ml in each case.

2.4 Estimation of Protein and Cholesterol

2.4.1 Determination of Protein by the Hartree Method

A few minor modifications to the method described by Hartree (1972) were introduced. Only half the volumes indicated by Hartree (1972) were used. In addition, for samples known to contain substances which interfere with the determination of protein by this method (such as catecholamines and hepes) the following procedure was adopted prior to the estimation of protein. Protein in each sample was precipitated by 0.5 ml ice cold 10% (w/v) TCA and left in ice for 10 minutes before the tubes were centrifuged. The supernatant was carefully removed, the insides of the tube being cleaned with tissue paper and the pellets resuspended in 3% NaOH, 2% sodium deoxycholate. Aliquots of this solution were then assayed for protein by the Hartree procedure (1972). Bovine serum albumin was used as standard.

2.4.2 Determination of Protein by the Dye Binding Method

Protein was also estimated by the coomassie brilliant blue G-250 dye binding method of Bradford (1976) using bovine serum albumin as standard. Samples were made up in 0.15 M NaCℓ and the scaled down version (microprotein assay) of the assay used. Cuvettes were cleaned with detergent, water and acetone as recommended.

2.4.3 Determination of Cholesterol

The commerciably available kit for the estimation of serum cholesterol supplied by BDH Limited was used. Cholesterol (5 mg/ml in butanol) was used as standard.

2.5 Other Methods

For other methods see individual methods sections of chapters 3-7.

CHAPTER 3

CHARACTERIZATION OF THE BOVINE ADRENAL CHROMAFFIN GRANULE MEMBRANE

CHARACTERIZATION OF THE BOVINE ADRENAL CHROMAFFIN GRANULE MEMBRANE

3.1 Introduction

The basis of the purification of chromaffin granule membranes is the ability of chromaffin granules to be separated from other organelles present in homogenates of bovine adrenal medullae. This is achieved because of the relatively higher density of the granules. Smith and Winkler (1967a) found that chromaffin granules could be recovered as a sediment following centrifugation through 1.6 M sucrose while mitochondria remained at the top of the 1.6 M sucrose layer and lysosomes either pelleted on top of the granules and could be removed by washing the pellet or were recovered in the 1.6 M sucrose layer. The use of larger volumes of 1.6 M sucrose (Helle et al., 1971) can further reduce the mitochondrial content of the granule preparation obtained. Schneider (1972) confirmed Smith and Winkler's (1967a) findings and also showed that granules purified in this way did not appear to be contaminated with cytoplasmic contents of adrenal medullary cells but observed that sedimenting the granules through 1.8 M sucrose removed more lysosomal and microsomal contamination than centrifugation through 1.6 M sucrose. This greater purity is, however, achieved at the expense of the yield.

Granule membranes can be prepared from purified chromaffin granules by centrifugation following lysis of the granules either by freeze-thawing (Winkler et al., 1970) or by hypotonic shock. Eagles et al. (1975) has shown that chromaffin granule membranes will vesiculate so that further washings of the membranes by resuspension and centrifugation should be accompanied by freeze-thawing or hypotonic shock to remove traces of soluble proteins trapped within these vesicles. The membranes can be further purified by continuous sucrose density

gradient centrifugation (Schneider, 1972; Phillips, 1973; Helle, 1973a; Muller and Kirshner, 1975) where they equilibrate at just above 1.0 M sucrose (Schneider, 1972; Phillips, 1973; Muller and Kirshner, 1975). Membranes of contaminating cell particles (mitochondria, microsomes) equilibrate at higher sucrose molarities.

I have isolated chromaffin granule membranes by three cycles of resuspension, hypotonic shock and centrifugation of granules purified by sedimentation through 1.8 M sucrose (Schneider, 1972). I, however, used 20 ml of 1.8 M sucrose as opposed to the 7 ml used by Schneider (1972). Granule membranes prepared in this or a similar manner have been shown to have a distinct polypeptide profile compared to membranes of the endoplasmic reticulum (Hortnagl, 1976), the golgi apparatus (Trifaro et al.,1976) and mitochondria (Winkler et al., 1970) and the soluble proteins of the chromaffin granule lysate (Winkler et al., 1970) as shown by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. They have also been shown to contain only low levels of marker enzymes for other membranes of the bovine adrenal medullary cell (Schneider, 1972).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis is the method of choice for separating membrane proteins from one another. Total solubilization and dissociation of membrane proteins and lipids can be achieved by incubating the membranes for fifteen minutes at 37^{o}C or one to two minutes at 100^{o}C with 1-2% SDS (Fairbanks et al., 1971; Laemmli, 1970; Weber and Osborn, 1969). Proteins denatured by SDS assume the configuration of a doubled over helical rod such that the length of the particle is proportional to the polypeptide chain length (Reynolds and Tanford, 1970a). Furthermore, saturation with SDS (1.4 g SDS is bound per g protein) swamps the intrinsic charge of the protein and results in the similiar charge per unit mass for protein-SDS complexes (Reynolds and Tanford, 1970b). Consequently the mobility of

protein-SDS complexes, as determined by SDS polyacrylamide gel electrophoresis is inversely proportional to the logarithm, to the base ten, of the molecular weight of the protein (Shapiro et al., 1967; Weber and Osborn, 1969).

Because of the large amount of bound SDS, protein-SDS complexes have high electrophoretic mobilities and this shortens the time required for electrophoresis. Lipid-SDS complexes migrate much faster than protein and do not usually interfere with the electrophoretic properties of protein-SDS complexes (Lopez and Siekevitz, 1973). Thus lipid extraction prior to electrophoresis of membrane samples is not necessary. Since SDS totally solubilizes membranes centrifugation of the detergent treated samples is also not required.

After SDS polyacrylamide gel electrophoresis protein can be detected in the gel by staining with one of a variety of dyes, e.g. amido black, Ponceau S and coomassie brilliant blue, the latter being the most sensitive. Staining is performed in acidified media in order to keep the proteins positive to bind this anionic dye. However, before this can be done SDS must be removed from the protein by washing the gel with 25% isopropanol (Fairbanks et al., 1971) or 45% methanol (Weber and Osborn, 1969). This also serves to 'fix' the proteins in the gel since SDS removal causes protein aggregation. In practice the stain can be included in the alcohol fixation stage. Stained gels can be destained with an acidic solution containing alcohol (Fairbanks et al., 1971; Weber and Osborn, 1969). A disadvantage of SDS polyacrylamide gel electrophoresis is that enzymes cannot be located in the gels by "activity staining" since most enzymes are irreversibly denatured by treatment with SDS.

In this chapter is reported the characterization of the chromaffin granule membrane by SDS polyacrylamide gel electrophoresis and enzyme assays. The cholesterol content and spectral properties of the membrane

have also been determined. Dopamine-β-hydroxylase (E.C.1.14.17.1), Mg^{++} activated ATPase (E.C.3.6.1.4), NADH:(acceptor) oxidoreductase (E.C.1.6.99.3) and phosphatidylinositol kinase (E.C.2.7.1.67) have all been shown to be present in the chromaffin granule membrane while analysis of the reduced versus oxidized difference spectrum has been interpreted as indicating the presence of a b-type cytochrome (see Introduction section 1.1.9). The membrane is also characteristically rich in cholesterol. These features of the granule membrane can be used to compare my preparation with those of other workers. In addition I have assessed contamination of the granule membranes with mitochondrial membranes and the granule lysate by comparing their gel patterns with that of chromaffin granule membranes as well as assaying the latter for monoamine oxidase (E.C.1.4.3.4), a marker enzyme for the outer mitochondrial membrane.

3.2 Materials and Methods

3.2.1 Materials

The Mg^{++} ATPase preparation was kindly donated by Dr. D.K. Apps, Department of Biochemistry, Edinburgh University and was purified from chromaffin granule membranes according to Apps and Glover (1978). Concanavilin A was obtained from Boehringer Corporation (London) Ltd., and Sepharose 4B from Pharmacia.

3.2.2 Concanavilin A - Sepharose Affinity Chromatography

15 g sepharose 4B was activated with cyanogen bromide according to the method of March et al. (1974). After washing with cold 0.1 M $NaHCO_3$ and then distilled water, the activated sepharose was filtered under vacuum to a moist cake and suspended in 10 ml 1 M NaCl, 0.06 M $NaHCO_3$ containing 0.12 g concanavilin A. The mixture was gently agitated in the cold for 24 hr before washing the coupled sepharose by

centrifugation with 0.7 M $NaHCO_3$ and then 1 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnSO_4$, 0.1 M acetate, pH 6 also in the cold.

Concanavilin A - sepharose affinity chromatography of the granule lysate was performed as described by Rush et al. (1974). The column size was 7.0 by 0.5 cm and the flow rat 10 ml per hr. Material eluted with α-methyl-D-mannoside was dialized against 10 mM hepes, pH 7 and concentrated by lyophilization.

Chromaffin granule membranes were solubilized with 0.5% (w/v) lubrol PX by incubation at 0°C for 30 min. Particulate material was removed by centrifugation at 160,000 g-av for 1 hr at 2°C. The solubilized membranes were then subjected to affinity chromatography as described above. When the partial purification of dopamine-β-hydroxylase was followed by enzymic assay 0.25 mg/ml catalase (34,000 sigma units/ml; 1 unit catalase decomposes 1 mole H_2O_2/min at pH 7, 25°C using H_2O_2 concentration of 10 mM) was included in all the buffers.

The concanavilin A - sepharose could be reused. After elution with α-methyl-D-mannoside the column was washed with 1 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnSO_4$, 0.1 M acetate, pH 6. When not in use concanavilin A - sepharose was stored at +4°C in this buffer with azide added as a preservative.

3.2.3 Preparation of Mitochondrial Membranes from Large Granule Fraction

The large granule fraction was subjected to continuous sucrose density centrifugation (Phillips, 1973). Fractions enriched in the mitochondrial marker enzyme monoamine oxidase were pooled and membranes prepared from them by hypotonic shocking and centrifugation as described for chromaffin granules (see section 2.1.2).

3.2.4 Other Methods

See chapter 2 for the preparation of chromaffin granule membranes and lysate, sodium dodecyl sulphate polyacrylamide gel electrophoresis,

enzyme assays, cholesterol and protein determinations, and the scanning of the reduced versus oxidized difference spectrum of granule membranes.

3.3 Results

3.3.1 Separation of Chromaffin Granule Membranes by SDS Polyacrylamide Gel Electrophoresis Under Reducing Conditions

3.3.1.1 Electrophoresis in 8% Acrylamide Gels

Fig. 3.1 shows a densitometric scan and Fig. 3.2 a photograph of an 8% (w/v) acrylamide gel containing chromaffin granule membranes separated by SDS polyacrylamide gel electrophoresis under reducing conditions and stained for protein with coomassie brilliant blue. Approximately 100 µgms protein has been applied to each lane of the gel. Other 8% acrylamide gel patterns of granule mebranes can be seen in Fig. 3.9 and 3.11 containing 50 - 150 µgms protein. Although the degree of resolution is variable, not every band seen in Fig. 3.2 can always be identified, major staining bands can usually always be located. Up to 53 bands have been identified between the top of the gel and the tracker dye. Lipid migrates ahead of the tracker dye and is poorly stained with coomassie brilliant blue.

The gels have been calibrated with molecular weight markers and the molecular weights calculated for each band, numbered in order of decreasing molecular weight, are listed in Table 3.1. Polypeptides of molecular weight c 18,000 daltons or less migrate with the tracker dye, bromophenol blue. These components can be resolved in gels of higher percentage acrylamide (see following section).

Very little stainable material fails to enter the gel indicating that the granule membrane is almost completely solubilized by boiling with sodium dodecyl sulphate and dithiothreitol. This ensures that the

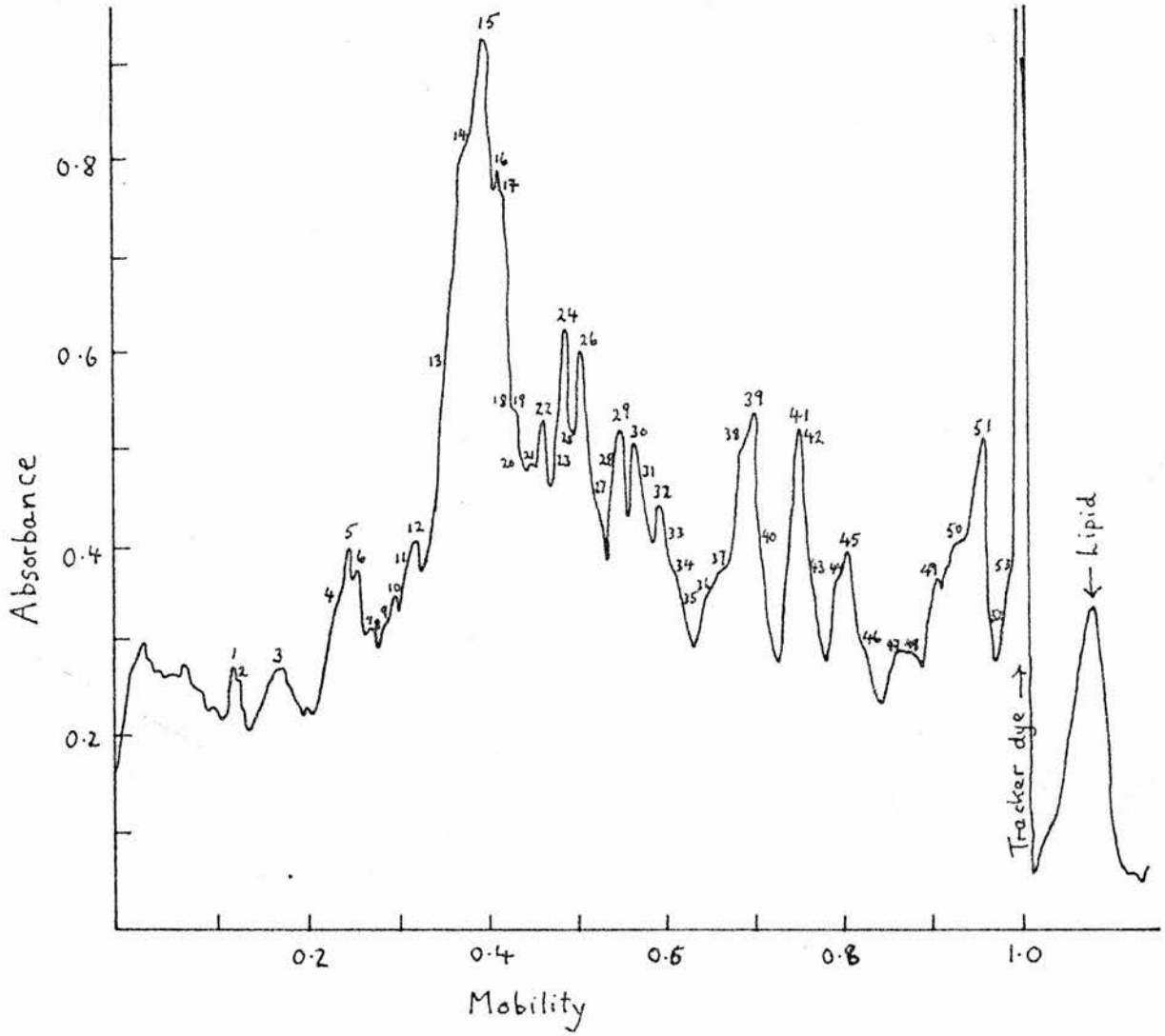
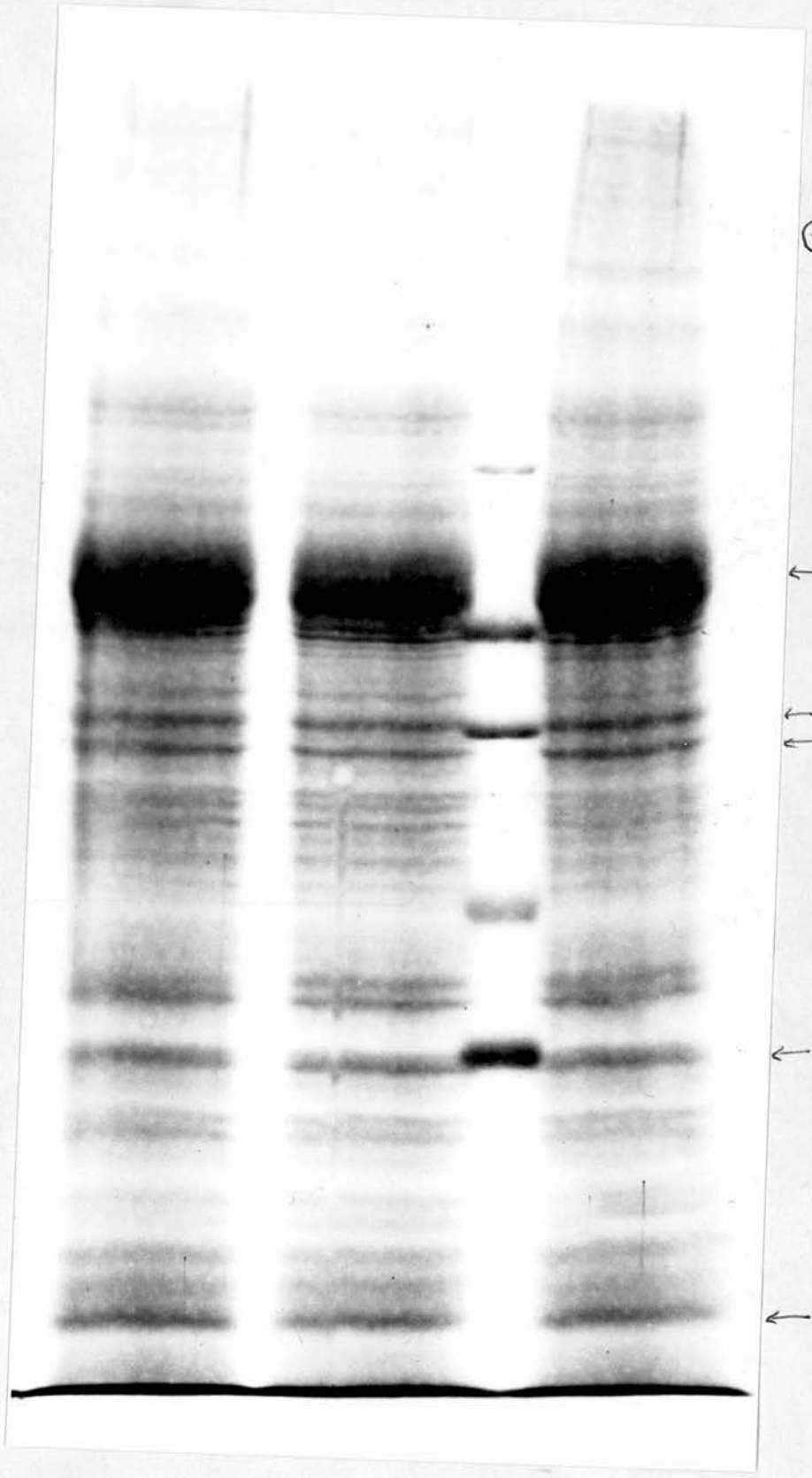


Fig. 3.1 Chromaffin Granule Membranes Separated by SDS Polyacrylamide Gel Electrophoresis Under Reducing Conditions in 8% (w/v) Acrylamide Gels

Purified chromaffin granule membranes (100 μgm protein) were separated by SDS polyacrylamide gel electrophoresis in 8% (w/v) acrylamide gels under reducing conditions. The gel was stained for protein with coomassie brilliant blue and then densitometrically scanned at 570 nm.

Fig. 3.2 Chromaffin Granule Membranes Separated by SDS Polyacrylamide Gel Electrophoresis Under Reducing Conditions in 8% (w/v) Acrylamide Gels

Photograph of part of an 8% (w/v) acrylamide slab gel containing granule membranes (0.1 mg protein/lane) in lanes 1, 2 and 4 and molecular weight markers in lane 3. SDS polyacrylamide gel electrophoresis was performed under reducing conditions and the gel was stained for protein with coomassie brilliant blue. The molecular weight markers used were phosphorylase a (reduced molecular weight 94,000 daltons), bovine serum albumen (68,000), catalase (60,000), H-chain γ globulin (50,000), ovalbumen (43,000), glyceraldehyde phosphate dehydrogenase (36,000) and L-chain γ globulin (23,500).


O
+
← 15
← 24
← 26
← 41,42
← 51
LANE 1 2 3 4

Band No	M. WT. x 10^{-3}		Staining Intensity in 8 % gel	Comments
	8% gel	8-20% gel		
1	137	165	weak	
2	134	160	weak	
3	123	142	v. weak	
4	107	120	weak	
5	104		moderate	
6	102			
7	98	110	v. weak	
8	97	106	v. weak	
9	94	97	weak	
10	91		weak	
11	89	93	moderate	
12	87		moderate	
13	82	77	v. strong	Dopamine-β-hydroxylase
14	78	72	v. strong	
15	74	68	v. strong	
16	71		strong	
17	70		strong	
18	69		weak	
19	68		weak	
20	66	67	v. weak	
21	65	66	v. weak	
22	63	65	moderate	
23	62	63	v. weak	
24	59	57	strong	
25	58	55	weak	
26	56	53	strong	
27	55	51	weak	
28	53	49	moderate	subunits of the Mg^{++} ATPase
29	52		moderate	
30	50	47	moderate	
31	50		weak	
32	48	46	moderate	
33	46	44	v. weak	

Band No	M. WT. x 10^{-3}		Staining Intensity in 8% gel	Comments
	8% gel	8-20% gel		
34	44	42	weak	
35	43	41	v. weak	
36	41		v. weak	
37	40	39	weak	
38	38	37	strong	
39	36	35	strong	
40	35	34	v. weak	
41	33	33	moderate	
42	33	31	strong	
43	31	30	weak	
44	29	28	moderate	Subunit of Mg^{++} ATPase
45	28	28	moderate	
46	26		weak	
47	25	26	weak	
48	24	26 24	weak	
49	23	24	moderate	Chromomembrin B. Relatively more heavily stained in gradient gels, especially 50 and 51
50	21	22	moderate	
51	20	21	strong	
52		21	weak	
53		20	moderate	
54		20	weak	
55		19	v. weak	
56		18	v. weak	
57		18	v. weak	
58		17	v. weak	
59		16	v. weak	
60		15	weak	
61		14	moderate	
62		14	v. weak	
63		13	v. weak	
64		12	v. weak	
65		11	v. weak	
66		11	v. weak	

Table 3.1 Polypeptides of the Chromaffin Granule Membrane

CBB staining pattern obtained is representative of the membrane as a whole.

The major staining region of gels of reduced chromaffin granule membranes occurs a third of the way down the gel at a molecular weight of 70 - 80,000 daltons. Only minor staining bands are observed between this region and the top of the gel. CBB stained bands 1 and 2 are the first consistently observable bands and usually migrate as a doublet. Higher molecular weight bands are seen but they are extremely variable in number, mobility and staining intensity. These may represent aggr-eggates of lower molecular weight components. Even under these condi-tions of electrophoresis dimers and trimers of bovine serum albumin still occur although the 68,000 dalton monomer is the major staining species. The broad band referred to as band 3 probably comprises sev-eral components whilst bands 4, 5 and 6 are variably stained and are not always as well resolved as in Fig. 3.1 and 3.2. Bands 7, 8, 9 and 10,which are faintly stained and clearly resolved as two doublets in Fig. 3.2, are not always seen and bands 11 and 12 sometimes merge into band 13.

As can be seen in the densitometric scan (Fig. 3.1) the major staining region of the gel comprises several components (bands 13, 14, and 15) which characteristically migrate as broad bands even when much smaller amounts of protein are applied to the gel. The molecular weights of bands 13, 14 and 15 are 82,000 daltons, 78,000 daltons and 74,000 daltons respectively. It is difficult to estimate the amount of membrane protein which these three components comprise. Quantita-tion of the relative amount of staining in this region of the gel is hampered by a characteristic uneven background staining (Fig. 3.1). If one assumes that bands 13, 14 and 15 contribute to this background

staining then these components account for 31% of the stainable material in the gel whereas their contribution is only 23% if they do not contribute to the background staining. In any case these calculations are only a rough guide to the amount of protein these components comprise in the membrane since they take no account of the protein migrating with the tracker dye and proteins vary in their ability to be stained with coomassie brilliant blue.

It may be that glycoproteins are responsible for or contribute to the high background staining. Glycoproteins migrate as extremely broad bands and are usually relatively poorly stained with coomassie brilliant blue. Gels of granule membranes have been shown to contain a number of glycoproteins (see chapter 4).

Migrating immediately ahead of CBB stained band 15 is a series of characteristically sharply resolved staining components (bands 16 to 19). These are more easily observed in photographs of gels rather than densitometric scans. Often these bands are missing and it is presumed that they have co-migrated with band 15.

The next region of the gel pattern of reduced granule membranes is occupied by bands 20 to 35 although it is likely that this crowded region of the gel contains more than 16 bands. Of these bands 24 and 26 are usually the most heavily stained although occasionally bands 28, 29 and 30 are more heavily stained. CBB stained bands 28 and 29 usually co-migrate but they run separately at lower protein loadings. Many minor components (bands 20, 21, 23, 25, 27, 31, 33 and 35) are present and can be seen as small shoulders on nearby major bands in the densitometric scans.

Bands 38 and 39, 41 and 42, and 44 and 45 occur as a series of doublets of moderate staining intensity. Each of these doublets is

associated with at least one minor staining component e.g. bands 36, 37, and 40 for 38 and 39; 43 for 41 and 42, and 46 for 44 and 45. Bands 47 and 48 are sometimes poorly stained compared to their staining in Figs. 3.1 and 3.2 while bands 49, 50 and 51 occasionally migrate with the tracker dye, or bands 49 and 50 form shoulders on band 51. Band 51 is variably stained. The last two bands, 52 and 53, are rarely seen and are presumed to co-migrate with the tracker dye when not observed.

3.3.1.2 Electrophoresis in 10% Acrylamide Gels

The gel pattern of granule membranes separated in 10% (w/v) acrylamide gels by SDS polyacrylamide gel electrophoresis under reducing conditions (see Figs. 3.3 and 3.4) is similar to that obtained with 8% (w/v) acrylamide gels except that smaller molecular weight components can be resolved from the tracker dye. Major CBB staining bands observed in 8% acrylamide gels could be identified in 10% acrylamide gels on the basis of their molecular weight, staining with coomassie brilliant blue and relative position in the profile (see Fig. 3.3). However, lower molecular weight components were extremely diffuse so much so that CBB stained bands 49 to 53 were only partially separated. Staining of these components also appeared to be poor. Thus it was decided to use acrylamide gradient gels to identify small molecular weight polypeptides of chromaffin granule membranes.

3.3.1.3 Electrophoresis in 8% - 20% (w/v) Acrylamide Gradient Gels

Thirteen bands (54 - 66) with faster mobility than band 53 are seen in gradient gels of reduced chromaffin granule membranes (Figs. 3.5 and 3.6). The smallest component, band 66, has a molecular weight of 10,500 daltons (Table 3.1). Travelling immediately ahead of band 66 is lipid so that smaller molecular weight components will be obscured. None of the thirteen bands is a major CBB staining component and,

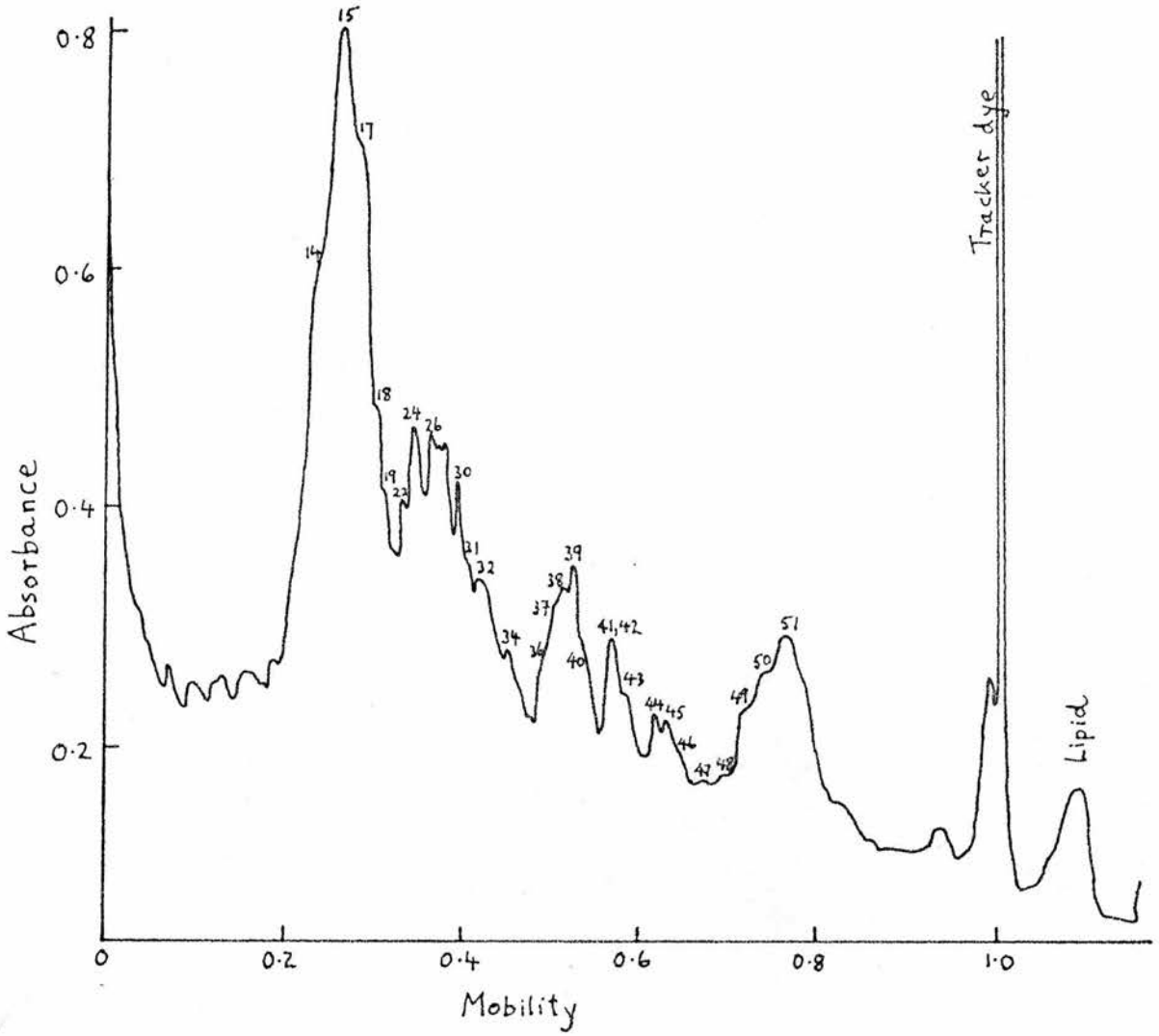


Fig. 3.3 Chromaffin Granule Membranes Separated by SDS Polyacrylamide Gel Electrophoresis Under Reducing Conditions in 10% (w/v) Acrylamide Gels

Densitometric scan of a lane of a 10% (w/v) acrylamide gel containing 100 μgms chromaffin granule membrane protein separated by SDS polyacrylamide gel electrophoresis under reducing conditions. The gel was stained for protein with coomassie brilliant blue.

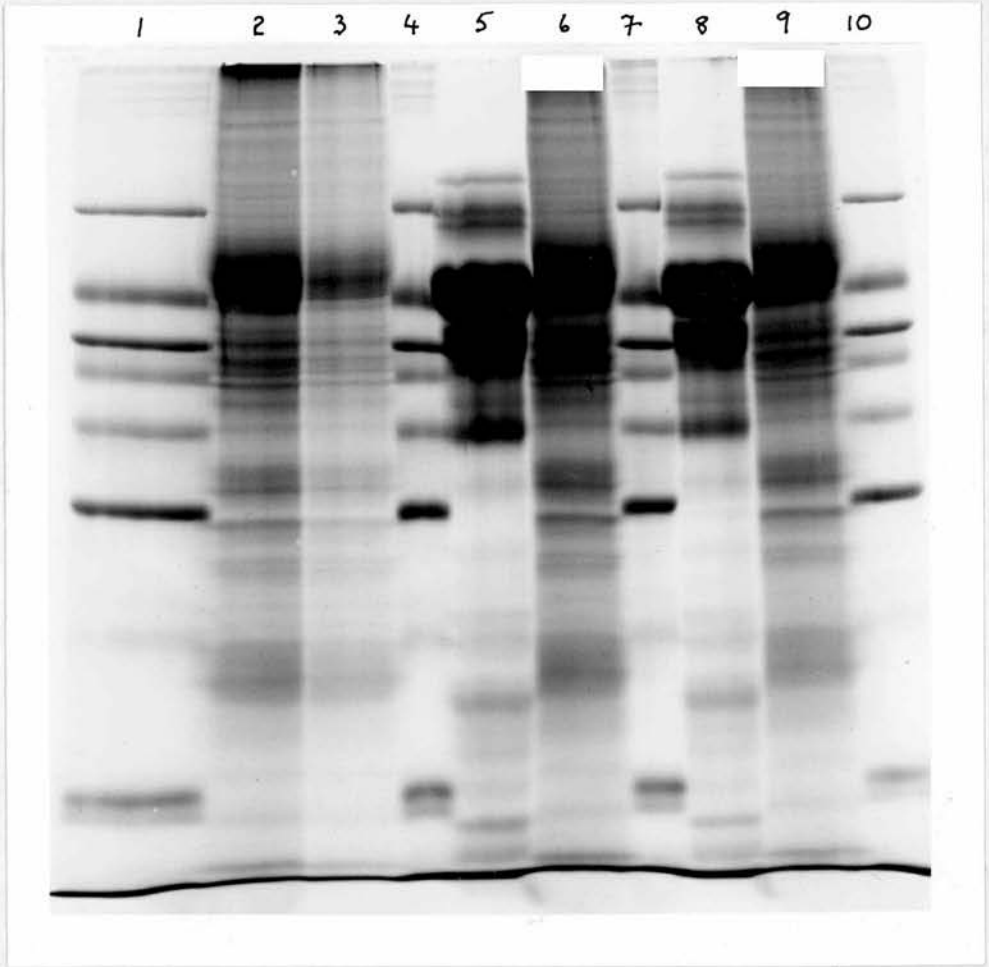


Fig. 3.4 SDS Polyacrylamide Gel Electrophoresis in 10% Acrylamide Gels Under Reducing Conditions

Lane 1, molecular weight markers; lane 2, chromaffin granule membranes (100 µgms protein); lane 3, chromaffin granule membranes (50 µgms); lane 4, molecular weight markers; lane 5, chromaffin granule lysate (100 µgms); lane 6, chromaffin granule membranes (100 µgm); lane 7, molecular weight markers; lane 8, chromaffin granule lysate (100 µgm); lane 9, chromaffin granule membranes (100 µgm); lane 10, molecular weight markers. Protein was detected by staining with coomassie brilliant blue. The molecular weight markers were phosphorylase a (94,000 daltons), bovine serum albumen (68,000), catalase (60,000), H-chain γ globulin (50,000), ovalbumen (43,000), glyceraldehyde phosphate dehydrogenase (36,000), L-chain γ globulin (23,500) and soya bean trypsin inhibitor (22,000).

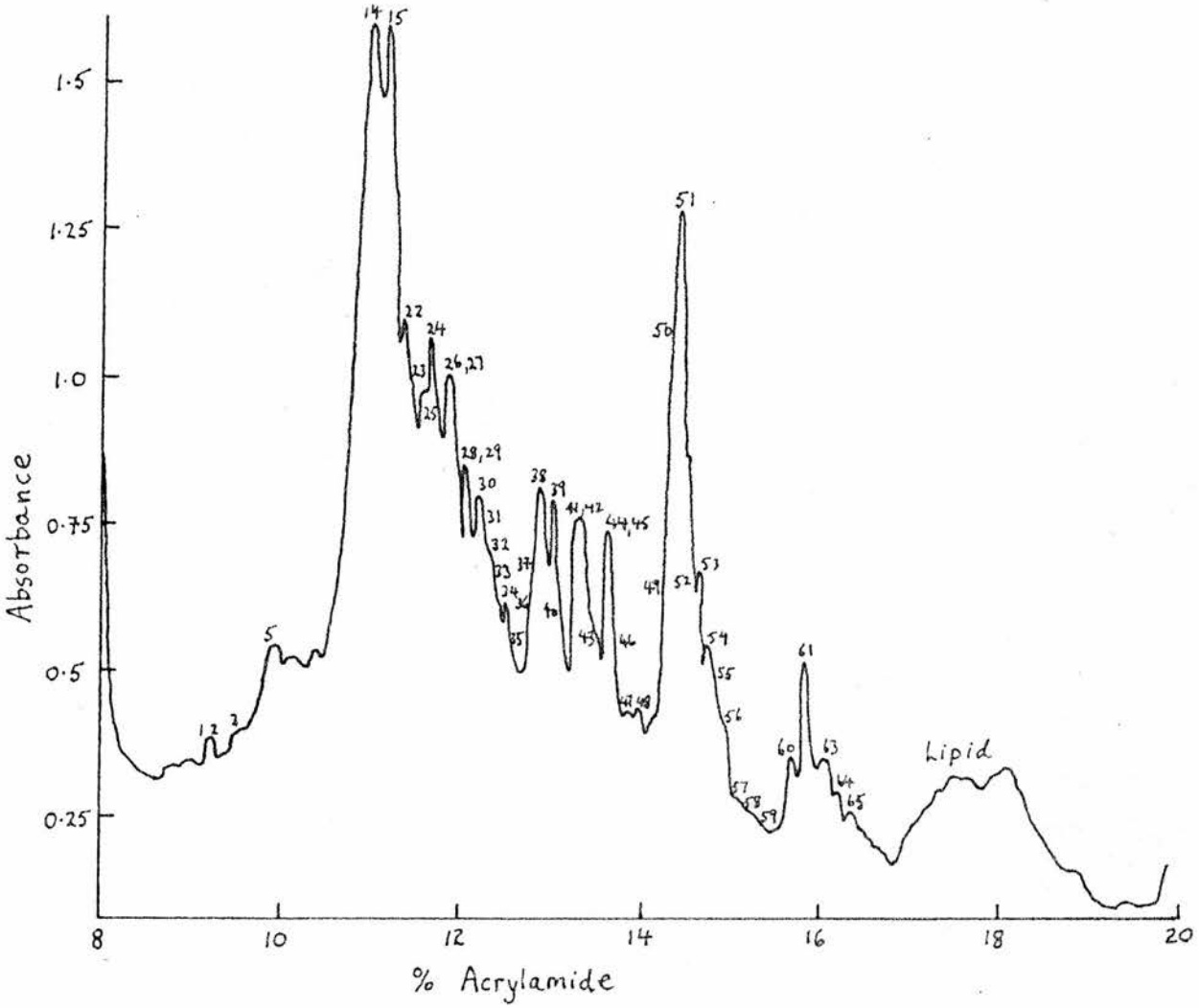


Fig. 3.5 Chromaffin Granule Membranes Separated by SDS Polyacrylamide Gel Electrophoresis in 8% - 20% Acrylamide Gradient Gels Under Reducing Conditions

A densitometric scan (570 nm) of a lane of an 8% - 20% acrylamide gradient gel containing chromaffin granule membranes (100 µgm) separated by SDS polyacrylamide gel electrophoresis under reducing conditions. The gel was stained for protein with coomassie brilliant blue.

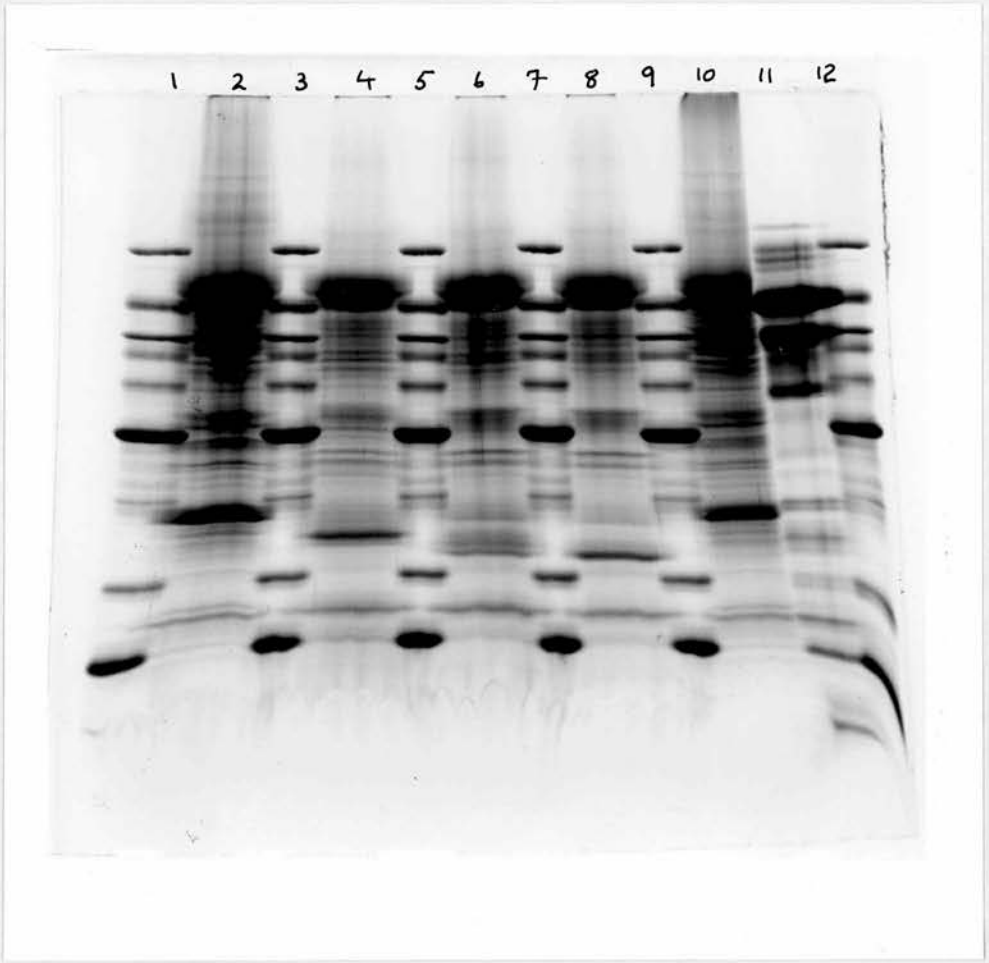


Fig. 3.6 SDS Polyacrylamide Gel Electrophoresis in 8% - 20% Acrylamide Gradient Gels Under Reducing Conditions.

Lanes 1, 3, 5, 7, 9 and 12 contain molecular weight markers; lanes 2 and 10 chromaffin granule membranes (100 µgms protein), lane 11, chromaffin granule lysate (100 µgm); lanes 4, 6 and 8, various pronase digested granule membrane samples (see chapter 5). The gel was stained with coomassie brilliant blue. The molecular weight markers were phosphorylase a (94,000 daltons), bovine serum albumen (68,000), catalase (60,000), H-chain γ globulin (50,000), ovalbumen (43,000), glyceraldehyde phosphate dehydrogenase (36,000), soya bean trypsin inhibitor (22,000) and cytochrome c (11,700).

unlike separation in 10% acrylamide gel, these components are sharply focused.

In Table 3.1 an attempt has been made to identify bands observed in gradient gels with those occuring in 8% acrylamide gels. This is not easy because molecular weights calculated from mobilities in the two gels are different, varying by 5%, and there also appears to be differences in the relative staining of certain bands. Thus the molecular weight of the major staining component of reduced granule membranes is 74,000 daltons in 8% acrylamide gels and 72,000 daltons in acrylamide gradient gels. Where many bands migrate closely estimates of their molecular weight are, therefore, not accurate enough to uniquely identify each band. CBB stained bands 50 and 51 are much more heavily stained with coomassie brilliant blue in gradient gels than in 8% acrylamide gels and now represent the second most heavily stained region of the gel. Despite these difficulties major staining bands in 8% acrylamide gels, bands 4, 5, 6, 13, 14, 15, 24, 26, 38, 39, 41,42, 44, 45, 50 and 51, have been identified in gradient gels of reduced granule membranes with a fair degree of certainty. This has been achieved mainly by use of relative staining and position in the gel pattern. Bands 13, 14 and 15 constitute 13% - 23% and bands 50 and 51 10% - 14% of the total stainable material in the gel. The lower figures represent the values calculated if one assumes that these bands do not contribute to the background staining whereas this contribution has been included to achieve the upper values (see section 3.3.1.1).

3.3.2 Separation of Chromaffin Granule Membranes by SDS Polyacrylamide Gel Electrophoresis Under Non-Reducing Conditions

The CBB staining pattern of an 8% acrylamide gel of non-reduced chromaffin granule membranes (Fig. 3.7 and 3.8) is markedly different

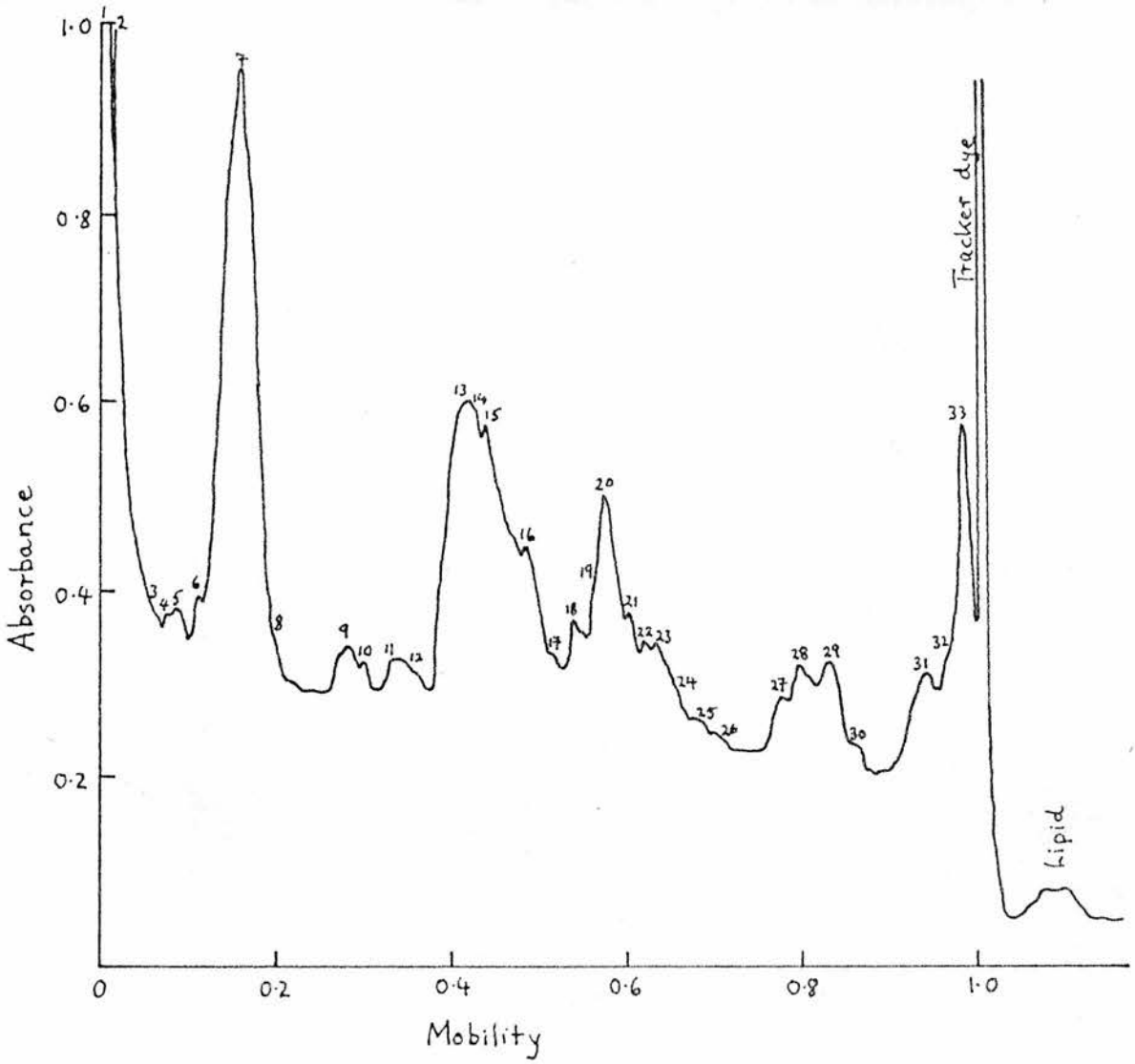


Fig. 3.7 Chromaffin Granule Membranes Separated by SDS Polyacrylamide Gel Electrophoresis in 8% (w/v) Acrylamide Gels Under Non-Reducing Conditions

100 μgm chromaffin granule membrane proteins were subjected to SDS polyacrylamide gel electrophoresis in 8% (w/v) acrylamide gels under non-reducing conditions. The gel was stained for protein with coomassie brilliant blue and scanned at 570 nm.

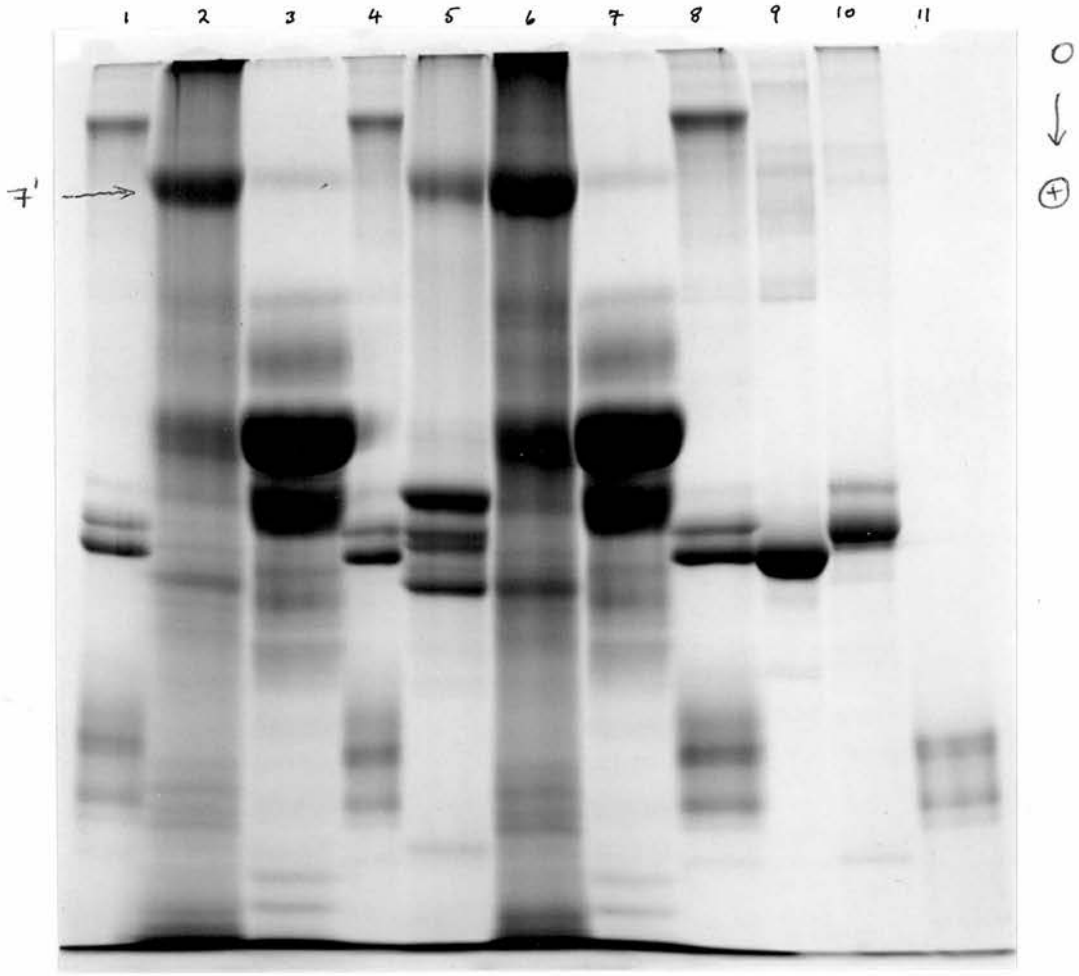


Fig. 3.8 SDS Polyacrylamide Gel Electrophoresis in 8% Acrylamide Gels Under Non-Reducing Conditions

Lane 1, 4, 8 molecular weight markers; lane 2 and 6, chromaffin granule membranes (100 μgm); lane 3 and 7, chromaffin granule lysate (100 μgm); lane 5, membrane bound dopamine-β-hydroxylase purified by concanavilin A - Sepharose affinity chromatography in the presence of catalase from lubrol PX solubilized granule membranes (50 μgm protein, see section 3.2.2), lane 9, bovine serum albumin (25 μgm); lane 10, catalase (20 μgm); lane 11, glyceraldehyde phosphate dehydrogenase (10 μgm). The gel was stained with coomassie brilliant blue following electrophoresis under non-reducing conditions.

from that of reduced granule membranes (Fig. 3.1 and 3.2). Much material fails to enter the gel and the most heavily stained band now migrates much nearer the top of the gel and has an apparent molecular weight of about 150,000 daltons. 33 non-reduced CBB stainable bands (1' to 33' - Table 3.2) are resolved in 8% acrylamide gels. In Table 3.2 an attempt has been made to identify non-reduced bands with reduced bands of the membrane. This is based purely upon identical mobilities. We can, however, identify non-reduced band 7' with reduced bands 13, 14 and 15 on the basis of their staining. Other bands of interest are non-reduced bands 31', 32' and 33' which have similar mobilities to reduced bands 49, 50 and 51 respectively. Band 33' is, however, relatively more heavily stained than band 51.

3.3.3 Comparison of Gel Patterns of Chromaffin Granule Membranes and Lysate and Mitochondrial Membranes

Mitochondria have been prepared from the large granule fraction by continuous sucrose density gradient centrifugation. Membranes were then prepared from them in an analogous manner to that used for the preparation of granule membranes from granules. This probably results in a membrane preparation of inner and outer mitochondrial membranes heavily contaminated with matrix proteins. SDS polyacrylamide gel electrophoresis in 8% acrylamide gels under reducing conditions reveals approximately 40 bands (Fig. 3.9). The pattern is completely different from that of chromaffin granule membranes. In particular the broad heavily stained bands 13, 14 and 15 characteristic of chromaffin granule membranes are missing from the mitochondrial membrane preparation. (There are bands in this region of gels of the mitochondrial membranes but these are much sharper). In addition the region of the gel containing the major staining bands of mitochondrial membranes contains

Band No.	Molecular Weight (daltons)	Staining Intensity	Reduced CBB bands with similar mobilities	Comments
1	170,000	v.strong		Material at top of gel
2	167,000	v.strong		
3	153,000	v. weak		
4	146,000	weak		
5	143,000	weak		
6	135,000	weak		
7	122,000	v.strong		DβH - non-reduced form
8	115,000	v.weak		
9	95,000	weak	6	
10	91,000	v.weak	7.8	
11	85,000	v.weak	12	
12	81,000	v.weak		
13	71,000	moderate	14?	Second major staining region
14	70,000	moderate	15?	similar mobility to Chrg A.
15	68,000	moderate	16,17	Could represent contamination
16	61,000	moderate	22	with Chrg A
17	57,000	v.weak	24,25	
18	54,000	weak	26,27	
19	52,000	weak		
20	50,000	moderate	28,29	Possibly a doublet
21	48,000	weak	30,31	
22	46,000	weak	32-35	
23	45,000	weak		
24	42,000	v.weak		
25	40,000	v.weak	36	
26	38,000	v.weak	37	
27	33,000	weak	41,42	
28	31,000	weak	43	
29	29,000	weak	44,45	
30	27,000	v.weak	46	
31	23,000	weak	49	Chromomembrin B. NR
32	22,000	weak	50	band 33 is relatively more
33	21,000	moderate	51	heavily stained than R 51

Table 3.2 Non-reduced Polypeptides of the Chromaffin Granule Membrane

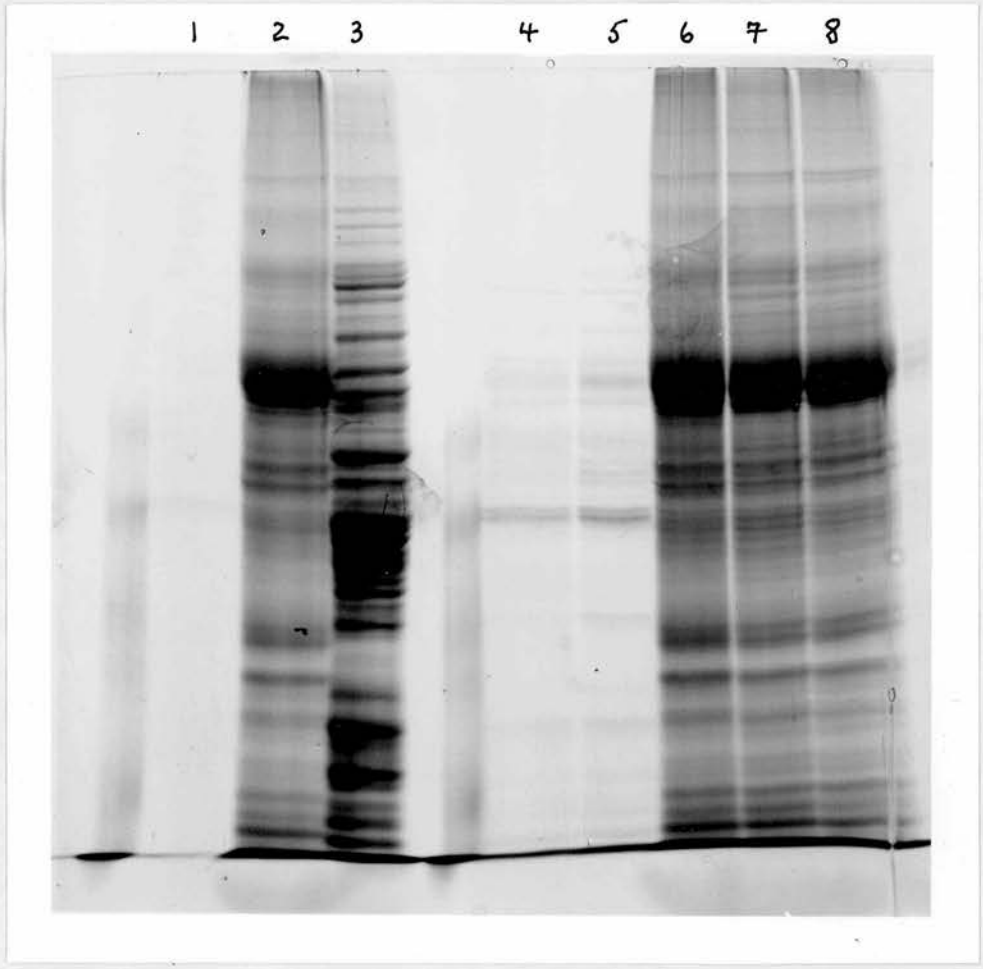


Fig. 3.9 Gel Patterns of Adrenal Medullary Mitochondrial Membranes and Semi-Purified Chromaffin Granule Membrane Mg^{++} ATPase

SDS polyacrylamide gel electrophoresis was performed in 8% (w/v) acrylamide slab gel under reducing conditions. Protein was detected by coomassie brilliant blue staining. Lane 1, Mg^{++} ATPase of chromaffin granule membranes partially purified from dichloromethane extracted membranes by velocity sedimentation in glycerol gradients (see Apps and Glover, 1978) - 3 µgm protein; lanes 2, 6, 7 and 8 chromaffin granule membranes (100 µgm protein); lane 3, mitochondrial membranes prepared by hypotonic shocking of mitochondria prepared from P_2 granules by continuous sucrose density gradient centrifugation (see section 3.2.3.). 100 µgm protein; lane 4 and 5, chromaffin granule membranes extracted with dichloromethane according to Apps and Glover, (1978) - 12.5 and 19.5 µgm protein respectively.

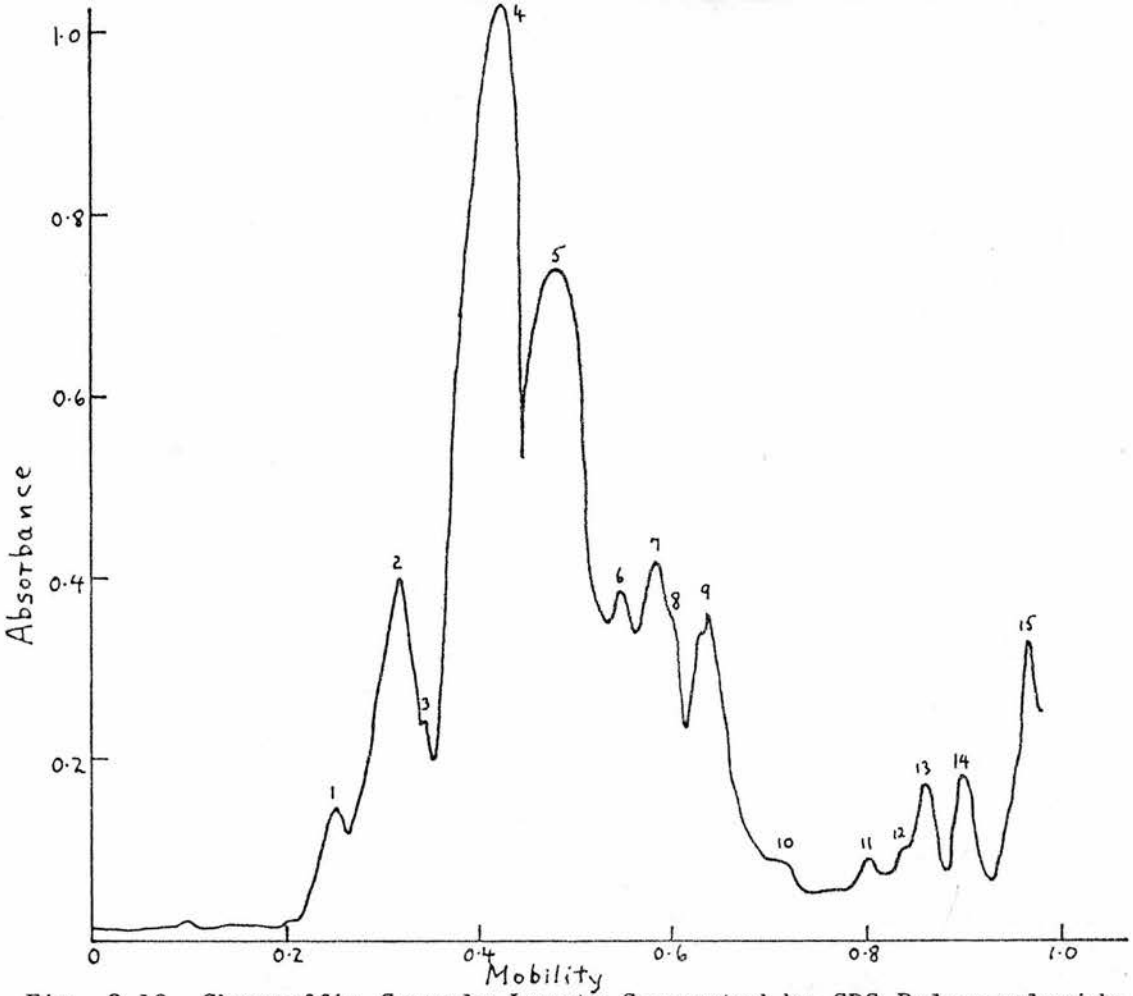


Fig. 3.10 Chromaffin Granule Lysate Separated by SDS Polyacrylamide Gel Electrophoresis in 8% Acrylamide Gels Under Reducing Conditions.

100 μgm chromaffin granule lysate protein was subjected to SDS polyacrylamide gel electrophoresis in an 8% acrylamide gel under reducing conditions. The gel was stained for protein with coomassie brilliant blue and densitometrically scanned at 570 nm.

only minor stained bands in gels of granule membranes.

Various gel patterns of the granule lysate can be seen in Fig. 3.4, 3.6, 3.10 and 3.11. The major CBB staining bands of gels of the matrix proteins are characteristically broad. Although the gel pattern is clearly different to that of chromaffin granule membranes the major stained bands of each profile have similar mobilities in 8% and 10% acrylamide gels. However, in acrylamide gradient gels the major staining component of the granule lysate clearly migrates ahead of the major staining components of granule membranes.

Unlike granule membranes gels of non-reduced and reduced granule lysate separated by SDS polyacrylamide gel electrophoresis are very similar, the chief difference being the appearance in non-reducing gels of a minor staining component having an identical mobility to the major stained band of non-reduced granule membranes (cf Fig. 3.8 and 3.11). Non-reducing and reducing gels of granule lysate proteins lack the characteristic background staining of CBB stained gels of granule membranes and do not show the diffussely stained component migrating ahead of the tracker dye and attributed to lipid which is present in granule membranes.

3.3.4 Identification of Dopamine-β-Hydroxylase and Mg^{++} ATPase in Gel Patterns of Chromaffin Granule Membranes

3.3.4.1 Dopamine-β-Hydroxylase

Dopamine-β-hydroxylase is the only concanavilin A binding protein of the chromaffin granule lysate (Rush et al., 1974; Aunis et al., 1975a) and can be purified by concanavilin A - sepharose affinity chromatography (Aunis et al., 1975a; Rush et al., 1974). When such a preparation of DβH is subjected to electrophoresis in the presence of SDS under reducing conditions (Fig. 3.11) the major band has a similar mobility

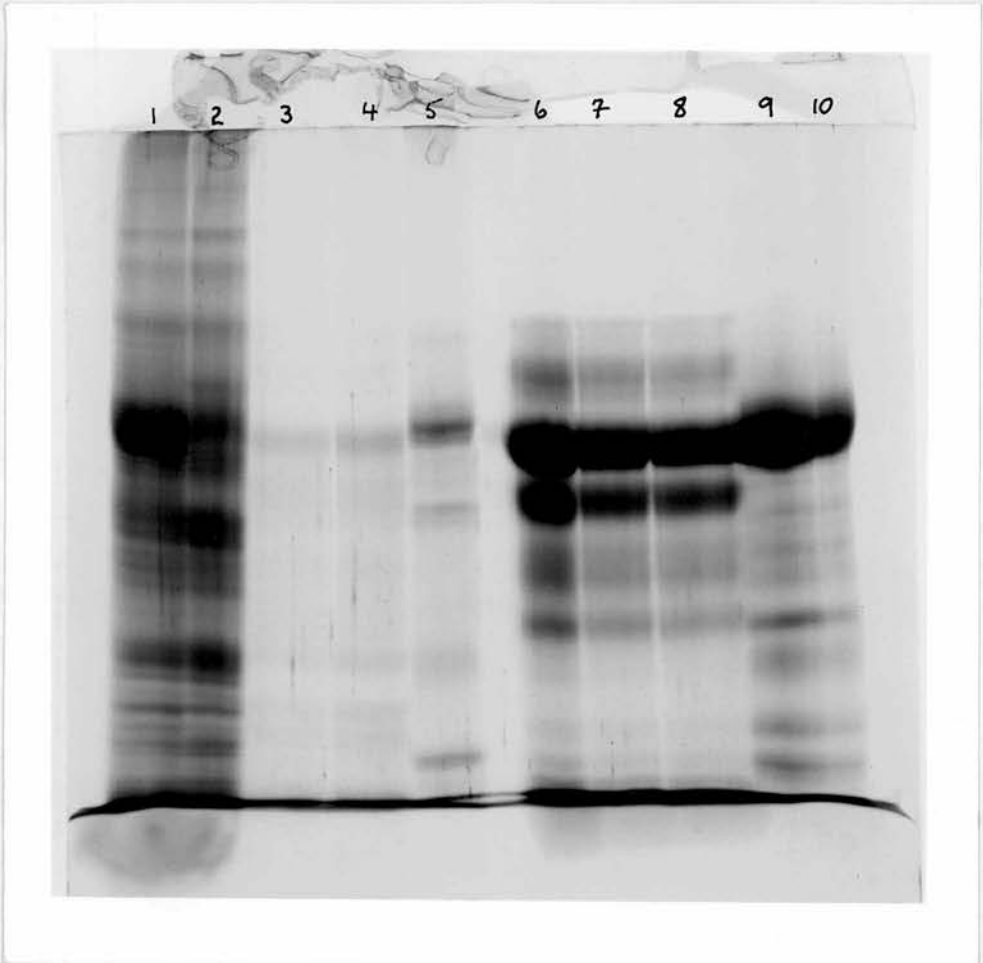


Fig. 3.11 Purification of Membrane Bound and Soluble Dopamine-β-Hydroxylase by Concanavilin A - Sepharose Affinity Chromatography

Lane 1, chromaffin granule membranes (100 μgm protein); lane 2, lubrol PX insoluble fraction of granule membranes; lane 3 and 4, lubrol PX solubilized chromaffin granule membranes (20μg); lane 5, membrane bound DβH purified from lubrol solubilized granule membranes by concanavilin A - Sepharose affinity chromatography (25 μgm); lane 6, chromaffin granule lysate (100 μgm protein); lane 7, 8 chromaffin granule lysate proteins which failed to adsorb to concanavilin A - Sepharose - 75, 50 μgm protein respectively; lanes 9, 10, soluble DβH purified by cancanavilin A - Sepharose affinity chromatography of granule lysate - 50, 25 μgm protein respectively.

to the major CBB staining component of the granule lysate. In non-reducing gels this band co-migrates with the slowest moving component of non-reduced gels of chromaffin granule lysate. Many minor CBB staining components are also present in the DβH preparation some of which have identical mobilities to granule lysate components (bands 9, 13, 14 and 15). Thus, there may be other concancavilin A binding components in the lysate or some of these bands may be degradation products of DβH. (In chapter 4 only DβH of the lysate proteins stains with FITC-con A although the majority of the major CBB staining bands of the lysate are not fixed in the gel prior to treatment with FITC-con A).

DβH can also be semi-purified by con A - sepharose affinity chromatography from lubrol PX solubilized chromaffin granule membranes. CBB stained bands 13, 14 and 15 of reduced gels of granule membranes (Fig. 3.11) are the major bands retained by the column and eluted by α-methyl-D-mannoside. Other bands of the membranes which are present in this preparation include bands 7, 8, 24, 26, 47 and 48 and a broad diffussely staining region co-migrating with bands 36-39. A concanavilin A binding glycoprotein has been identified (see chapter 4) in the region of bands 36 - 39 by FITC-con A staining although it does not appear to be these bands. Bands 24, 26, 47 and 48 also bind FITC-con A following separation by SDS polyacrylamide gel electrophoresis. Under non-reducing conditions of electrophoresis the major band of the membrane bound DβH preparation co-migrates with non-reduced CBB stained band 7' of granule membranes.

If catalase is included throughout the purification procedure then DβH can be assayed during purification (see Table 3.3). Approximately 50% of the enzyme activity could be solubilized with lubrol PX. A 2·34-fold purification of membrane bound DβH was achieved.

	Specific Activity	Enrichment	Total Activity	Recovery
Granule membranes	27.72	1	304.9	100%
Lubrol supernatant	9.43	0.34	127.4	41.8%
DβH from con A column	64.85	2.34	43.5	14.3%

Table 3.3 Purification of Membrane Bound Dopamine-β-Hydroxylase

For details see text. All activities are expressed as nmole tyr converted per min.

3.3.4.2 Mg^{++} ATPase

Purified Mg^{++} ATPase was a gift from Dr. D.K. Apps and was prepared by glycerol sedimentation velocity gradient centrifugation following dichloromethane extraction of chromaffin granule membranes (Apps and Glover, 1978). SDS polyacrylamide gel electrophoresis under reducing conditions reveals 3 bands in this preparation (Fig.3.9) corresponding to bands 28, 29 and 44 of reduced granule membranes which have molecular weights of 53,000 daltons, 52,000 daltons and 29,000 daltons respectively (see Table 3.1). These components (α,β and δ subunits of the Mg^{++} ATPase) had molecular weights of 51,000, 50,000 and 28,000 daltons respectively according to Apps and Glover (1978).

3.3.5 Monoamine Oxidase Activity of Chromaffin Granule Membranes

Monoamine oxidase (MAO) is an enzyme marker for the outer membrane of mitochondria. Chromaffin granule membranes, prepared according to the scheme outlined in Fig. 3.12, retained only 0.6% of the total monoamine oxidase activity of the 550 g-av supernatant at a specific activity of 0.063 nmole tyramine converted/min/mg protein at 37°C (tyramine concentration 22.7 μM). The granule membrane preparation showed no enrichment in MAO activity as would be expected if this enzyme were a component of the granule membrane. The majority of the mitochondria could be removed by differential centrifugation and sucrose density centrifugation. Hypotonic lysis of granules and washing of granule membranes with 10 mM hepes, pH 7 failed to remove any of the remaining mitochondrial contamination.

3.3.6 Cholesterol Content of Chromaffin Granule Membranes

Cholesterol is a characteristic component of chromaffin granule membranes (see chapter 1). When cholesterol is assayed with cholesterol oxidase using the assay kit supplied by BDH the granule membranes contain

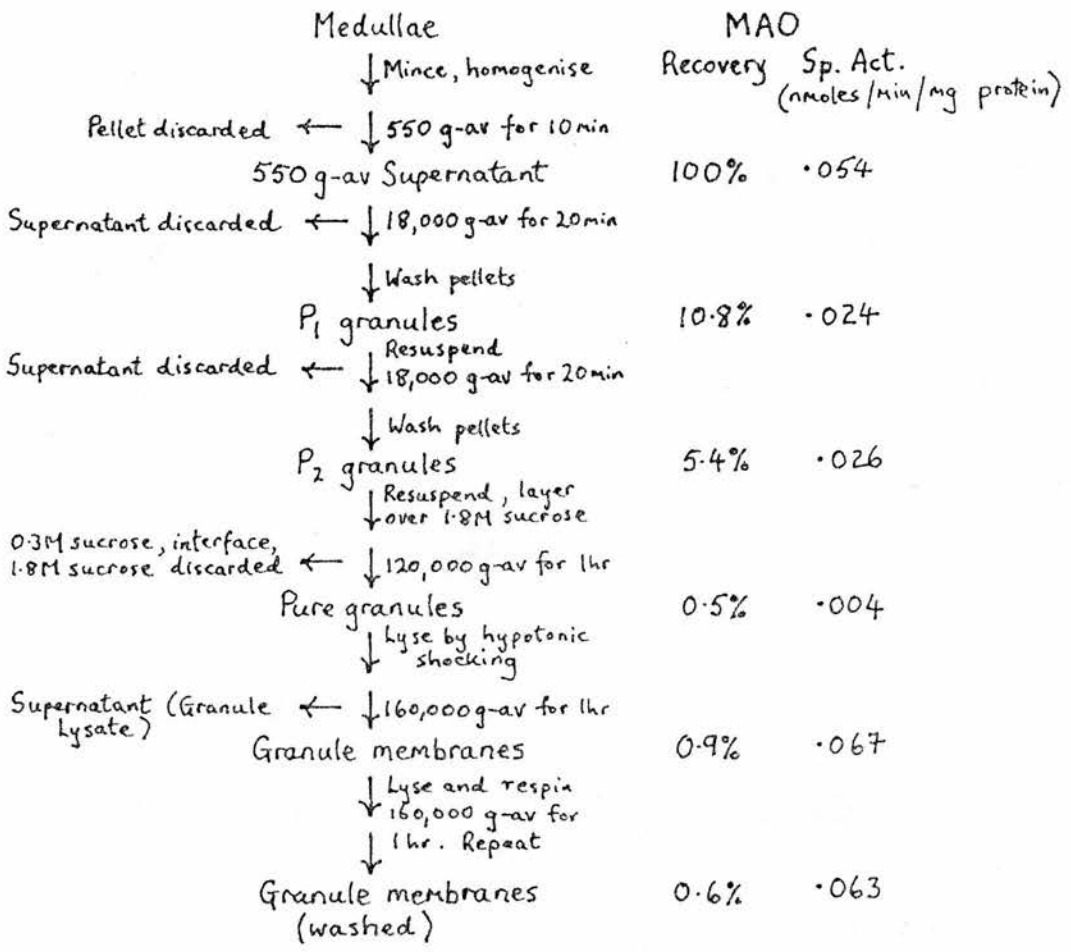


Fig. 3.12 Preparation of Chromaffin Granule Membranes and Recovery of Monoamine Oxidase

The preparation of chromaffin granule membranes is schematically outlined and the recovery and specific activity of monoamine oxidase is recorded at each stage.

approximately 0.9 mg cholesterol per mg protein. Granule membranes were prepared from granules sedimentated through 1.8 M sucrose by 3 cycles of hypotonic shocking and washing with 10 mM hepes, pH 7.

3.3.7 Enzyme Activities of Chromaffin Granule Membranes

When measured with a substrate concentration of 0.23 mM tyramine dopamine-β-hydroxylase of the membranes had a specific activity of 11.13 nmoles tyramine converted per min per mg protein at 37°C. DβH was assayed in the range where its activity is proportional to the protein concentration. It was found that the enzyme was unstable to storage at -20°C and/or freeze-thawing. Thus a preparation of granule membranes exhibited decreasing activities of 10.62, 5.55 and 3.6 nmoles tyramine converted/mg protein/min each time being assayed after a period of storage at -20°C.

The Mg^{++} activated ATPase of the membrane catalyzed the release of 48.49 nmoles of Pi from ATP per min per mg protein at 37°C using 1 mM ATP and 1 mM $MgCl_2$ while the activity of the NADH:ferricyanide oxidoreductase of the membranes was 0.12 μmoles NADH oxidized/min/mg protein at room temperature. The phosphatidylinositol kinase activity of granule membranes (0.46 nmoles Pi incorporated/mg protein/min at 37°C) represents the difference between the incorporation of ^{32}P into the membrane with and without EDTA included in the assay mix.

3.3.8 The Reduced versus Oxidized Difference Spectrum of Chromaffin Granule Membranes

In Fig. 3.13 is shown the reduced versus oxidized difference spectrum of chromaffin granule membranes. The dithionite reduced membranes showed absorption maxima at 428 nm, 528 nm and 559 nm. Occasionally a broad negative peak was observed in the region 440-450 nm. The difference in absorption between reduced and non-reduced granule membranes at 559 nm was 0.11 absorbance units/mg protein (3ml volume; 1 cm pathlength).

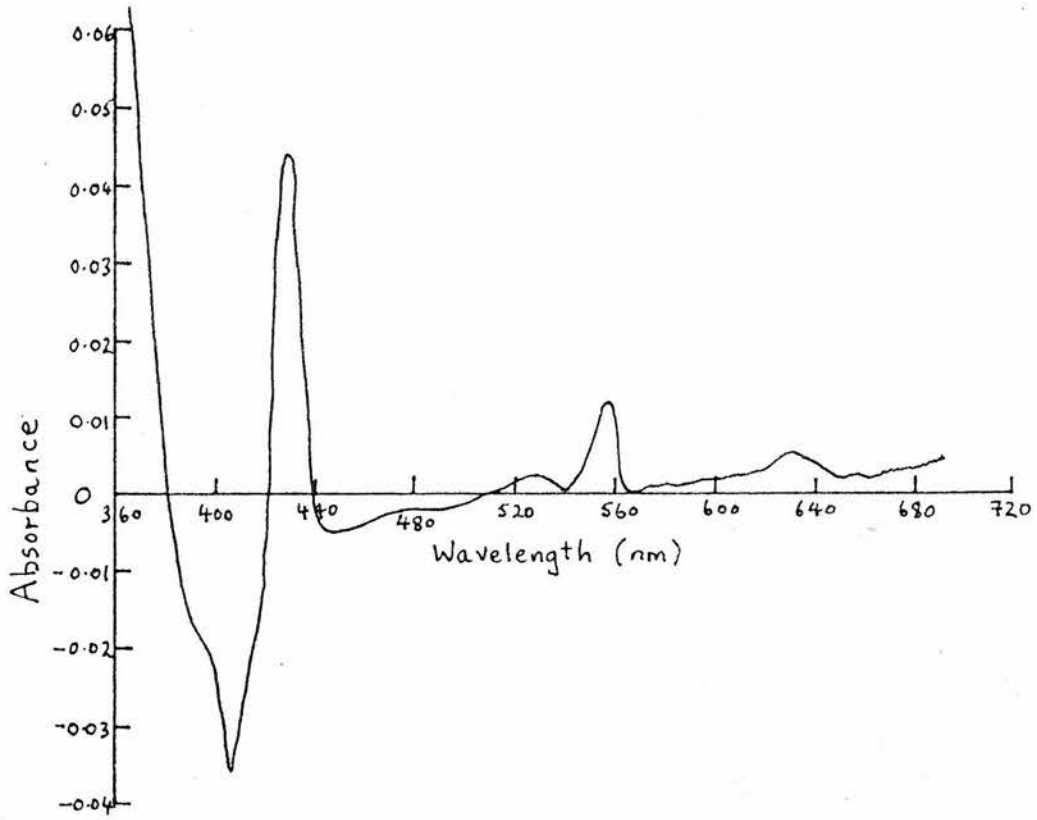


Fig. 3.13 The Reduced Versus Oxidized Difference Spectrum of Chromaffin Granule Membranes

Chromaffin granule membranes were reduced with dithionite. Untreated membrane samples were assumed to be fully oxidized. Each cuvette contained 0.1 mg protein suspended in 10 mM hepes, pH 7.

3.4 Discussion

3.4.1 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Chromaffin granule membranes have been separated under both reducing and non-reducing conditions of sodium dodecyl sulphate polyacrylamide gel electrophoresis. In the absence of reducing agents much protein fails to enter 8% acrylamide gels which may indicate that the membrane is incompletely solubilized by sodium dodecyl sulphate treatment alone. Whilst the presence of this material could be due to other reasons it is clear that a large proportion of the membrane proteins are being excluded from the analysis. Thus for analysis of the membrane proteins of the chromaffin granule membrane reducing conditions of SDS polyacrylamide gel electrophoresis have been preferred. Gel patterns of non-reduced membranes can, however, be useful in specific instances (see later).

Failure to reduce disulphide bonds makes the relationship between stained bands in gels and actual membrane polypeptides more uncertain. The same polypeptide could occur in the gel in several different aggregated states. Fully reduced polypeptides are also required for accurate molecular weight determination since the presence of intramolecular disulphide bands may prevent the double helical rod conformation of SDS - protein complexes to be formed. On the one hand the smaller hydrodynamic size that results will anomalously enhance mobility (Fish et al., 1970) whilst on the other hand the diminished SDS binding will lead to a diminished charge density and lesser mobility (Pitt-Rivers and Impiobato, 1968). In the case of bovine serum albumen the former factor is clearly the dominating one since bovine serum albumen migrates much faster under oxidizing rather than reducing conditons of electrophoresis.

The inclusion of reducing agents during SDS polyacrylamide gel electrophoresis does not, however, overcome the following problems: one stained band may comprise two or more polypeptides having similar or identical mobilities; certain proteins, especially small molecular weight hydrophobic polypeptides may be lost from the gel following electrophoresis; glycoproteins, especially heavily glycosylated ones may not stain at all with coomassie brilliant blue; disulphide aggregates can still occur (cf bovine serum albumen); and finally many proteins may be degraded during solubilization with SDS - denatured proteins are extremely susceptible to contaminating proteases. The last two problems can be overcome, partially at least, by using dithiothreitol, a much stronger reducing agent than the commonly used β-mercaptoethanol, and solubilizing samples with high concentrations of SDS (10%; w/w) and by incubation in a boiling-water bath in order to rapidly inactivate contaminating proteases.

Variations in mobility can also occur in reducing gels. In complex gel patterns the mobility of a particular component may be dependent on the presence of other components especially where a minor band migrates immediately in front of a major one (Fairbanks et al., 1971). Because of diminished SDS binding caused by the sugars present glycoproteins migrate anomalously (see chapter 4).

I have preferred to use slab gels as opposed to tube or disc gels. The former gel is generally considered to provide a greater degree of resolution than the latter - mainly because of the greater length over which components can be separated. "Edge" effects cause bands in disc gels to be curved upwards near the edge of the gel. In the slab gels used here up to 13 different samples can be electrophoresized at the same time in the one slab gel and only 2 of these samples (the ones in the

outer lanes) are likely to suffer from edge effects. The two outer lanes can, of course, be left blank. Artefacts can also arise in disc gels if the gels are not stained properly. Staining for too short a time will cause only the outside of the gel to be stained and rings of staining could be interpreted as two bands instead of one. Slab gels are thinner than disc gels and it is much easier for any stain to penetrate the gel completely. For comparative purposes slab gels are obviously much more ideally suited than disc gels. Two samples whose electrophoretic patterns are to be compared will be electrophoresized, stained and destained under identical conditions by virtue of being present in the same gel.

3.4.2 Separation of reduced chromaffin granule membranes by SDS polyacrylamide gel electrophoresis.

Sixty six coomassie brilliant blue staining bands have been identified in gels of chromaffin granule membranes separated by SDS polyacrylamide gel electrophoresis under reducing conditions. This number of components has been compiled from analyses of solubilized granule membranes resolved in 8% and 10% acrylamide gels and gradient gels of 8 - 20% acrylamide. The majority of the band observed are relatively minor staining components. In gradient gels two regions of the gels containing components with apparent molecular weights in the range 70 - 80,000 and 20 - 25,000 daltons account for most of the stainable material in the gel although in 8 and 10% acrylamide gels the lower molecular weight region is much less intensely stained.

Other workers have reported fewer bands in reduced granule membranes than the number observed here. Kirshner (1974) reported 15 bands, Eagles et al., (1975) detected 13 bands and Cahill and Morris (1979) observed

22 bands while from the densitometric scans of Huber et al. (1979) and Jockusch et al. (1977) gels of reduced granule membranes can be estimated not more than 15 and 35 components respectively. These gel patterns have been reproduced in Fig. 3.14 and are similar in broad outline at least to my gel pattern of the membranes. The increased resolution that I have obtained can be accounted for by the use of slab gels, choice of conditions for electrophoresis, optimal loading of gels with protein and, as mentioned above, the use of different gel concentrations.

Cahill and Morris (1979) and Jockusch et al. (1977) used slab gels and they also observe more components than groups which have used disc gels. The use of shorter gels and failure to catalogue minor staining bands may account for the fewer components reported by Cahill and Morris (1979) who used similar sample preparation and electrophoresis conditions to those employed by myself. Huber et al. (1979) found that more components of non-reduced granules could be resolved by electrophoresis in 8% acrylamide gels according to Fairbanks et al. (1971) than in 5.6% acrylamide gels using conditions described by Weber and Osborn (1969). The latter conditions were those used to separate reduced granule membranes as reported by Huber et al. (1979). Eagles et al. (1975) deliberately overloaded their gels so they could be stained for carbohydrate by the periodic acid - Schiff method which is less sensitive than coomassie brilliant blue staining for protein. This causes bands to broaden and obscure minor staining components which have similar mobilities.

Contamination of chromaffin granule membranes with other membranes of adrenal medullary cells is unlikely to be the cause of the greater number of bands I have observed in gels of reduced granule membranes. Other workers, using similarly prepared chromaffin granule membranes have shown them to possess a distinct polypeptide pattern, as shown by SDS polyacrylamide gel electrophoresis, from other organelle membranes of the adrenal medulla (see Introduction to this chapter). In addition I have

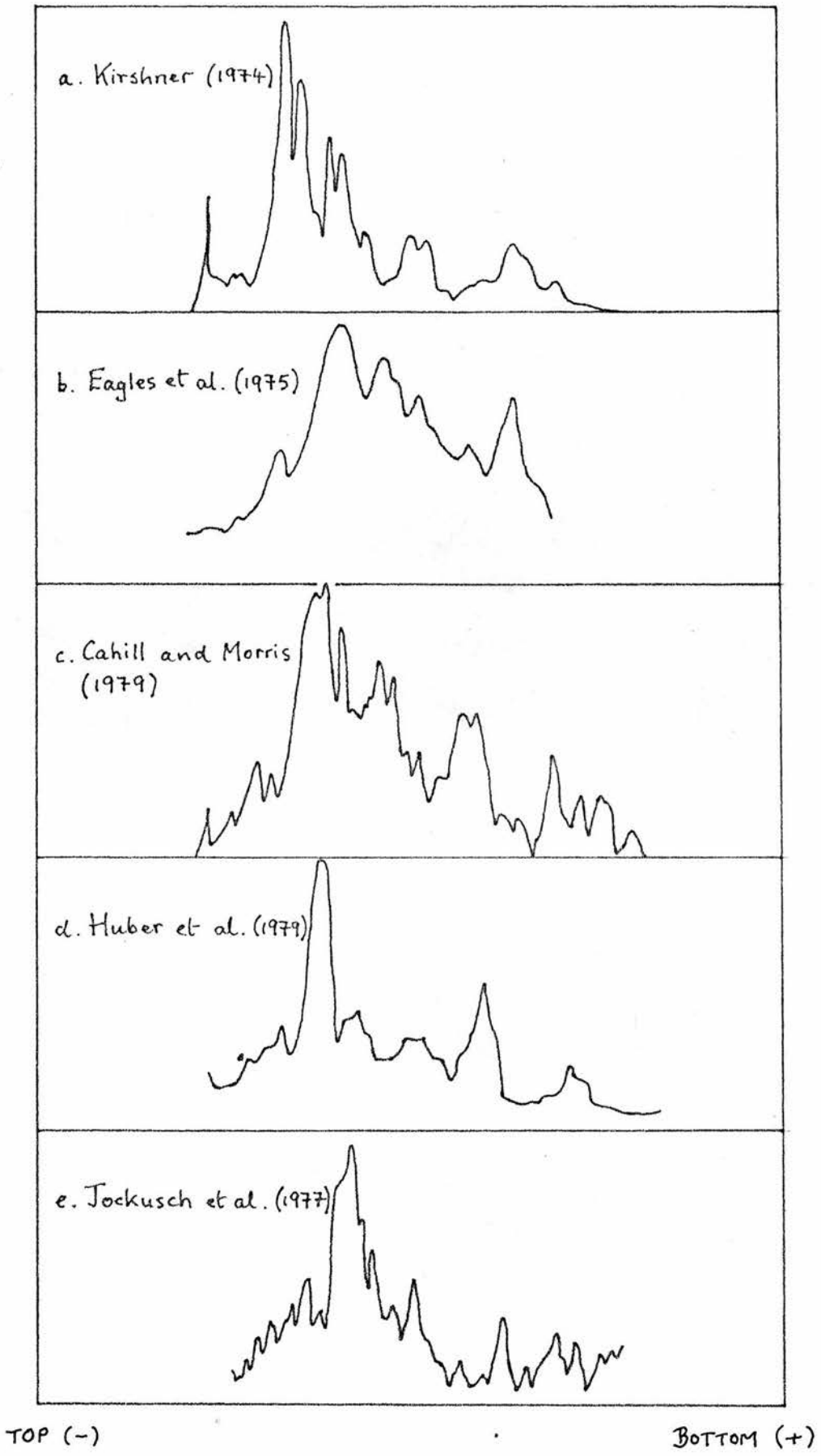


Fig. 3.14 Previously Published Gel Patterns of Reduced Chromaffin Granule Membranes

prepared mitochondrial membranes and found their gel pattern to be totally different from gels of granule membranes purified by myself. Consistent with this last observation is the occurrence of only low activities of mono amine oxidase, a marker enzyme for the outer membrane of mitochondria, in my granule membrane preparations.

Whilst gel patterns of the soluble proteins of the granule and the membrane proteins are also distinct traces of chromogranin A, the major protein component of the granule lysate, were usually observed in granule membrane preparations (Winkler et al., 1970; Kirshner, 1974). The source of chromogranin A in the granule membranes is likely to be contamination from the soluble content of the granules (Winkler et al., 1970; Kirshner, 1974). Helle has, however, claimed that chromogranin A is the major protein of the granule membrane (Helle and Serck-Hanssen, 1969; Helle, 1971a). This claim is based upon immunological studies and since the antisera used by Helle cross reacts with dopamine-β-hydroxylase it is, perhaps not so surprising that a high titre of antigen is found in the membranes.

I also find that gel patterns of soluble and membrane proteins of chromaffin granules are different. However it is difficult to assess possible contamination of granule membranes with chromogranin A in reduced gels under the conditions of electrophoresis I have employed since DβH, the major component of granule membranes, migrates with a similar mobility to that of chromogranin A. These proteins are clearly separated in non-reducing gels and I estimate that chromogranin A can account for no more than 10% of the membrane proteins.

Chromaffin granule membranes may, however, be contaminated by specific cytoplasmic proteins which have become adsorbed to the membrane. Phillips and Slater (1975) and Jockusch et al. (1977) have shown that the

inclusion of 0.15 M KCl during the preparation of granule membranes leads to the intensification of certain bands (actin and α-actinin) in gel patterns of the membranes. Variable protein adsorption could also explain the variability in staining, especially of minor bands, of components in gels of chromaffin granule membranes. The intrinsic variability of the gel method, lability of the protein components or weakness of their attachment to chromaffin granules could also account for alterations in relative staining intensity.

The application of two dimensional polyacrylamide gel electrophoresis to separate membranes previously studied by single dimensional methods (Rubin and Miłkowski, 1978) has shown many more components than originally identified. This, then is consistent with my belief that the greater number of bands I observe in gels of chromaffin granule membranes compared to those of other workers is due to better resolution rather than any differences in membrane preparations such as increased levels of contamination.

3.4.3 Separation of non-reduced chromaffin granule membranes by SDS polyacrylamide gel electrophoresis.

In 8% acrylamide gel thirty three bands can be seen when non-reduced granule membranes are separated by SDS polyacrylamide gel electrophoresis. As expected (see above) this number of staining components is in excess of the 21 bands observed by Cahill and Morris (1979), 11 by Bartlett and Smith (1974), 6 - 8 by Trifaro et al. (1976), 10 by Winkler et al. (1970) and the 20 or so peaks which can be identified in the scan shown in Fig. 3 of Huber et al. (1979).

As discussed earlier, despite the claims of other workers that non-reducing conditions of electrophoresis allows one to look at oligomers as opposed to polypeptides failure to break disulphide bands prevents

much protein from entering the gels and being analyzed. Consequently more emphasis has been placed upon separation of chromaffin granule membranes under reducing conditions of gel electrophoresis. Non-reducing conditions can, however, be used to confirm the identity and labelling of dopamine-β-hydroxylase and chromomembrin B in gels of granule membranes as well as assessing contamination of the membranes with chromogranin A.

3.4.4 Identification of Dopamine-β-Hydroxylase in Gels of Granule Membranes and Lysate Separated by SDS Polyacrylamide Gel Electrophoresis.

Based upon the following observations, dopamine-β-hydroxylase has been identified as CBB stained bands 13, 14 and 15 in reducing gels and band 7 of non-reducing gels of chromaffin granule membranes separated by SDS polyacrylamide gel electrophoresis. DβH is the major CBB staining component in gels of granule membranes (Hörtnagl et al., 1972) and has a molecular weight of 75,000 daltons under reducing conditions (Wallace et al., 1973; Aunis et al., 1974a; Kirshner, 1974; Craine et al., 1973) and 150,000 daltons when oxidized (Cahill and Morris, 1979) as indicated by its mobility when subjected to SDS polyacrylamide gel electrophoresis. It is a glycoprotein, the purified protein can be stained by the periodic acid - Schiff procedure following SDS gel electrophoresis (Wallace et al., 1973) and can be extracted from solubilized granule membranes by affinity chromatography using concanavilin A coupled to sepharose (Aunis et al.,1975a; Huber et al., 1979). The major CBB staining component of reducing and non-reducing gels of chromaffin granule membranes can also be stained by the periodic acid - Schiff stain (Eagles et al., 1975; Huber et al., 1979) and with dansyl hydrazine

and various lectins coupled to fluorescein isiothiocyanate (Cahill and Morris, 1979; see chapter 4).

DβH also occurs in the granule lysate but is only a minor constituent as shown immunologically (Hörtnagl et al., 1974) or by CBB staining (Hörtnagl et al., 1974; Kirshner, 1974; Smith et al., 1970) and is the slowest moving component in non-reducing gels of the soluble proteins of the granule (Smith et al., 1970; Hörtnagl et al., 1974). The membrane bound and soluble forms of this enzyme have similar properties both are glycoproteins (Aunis et al., 1974; Rush et al., 1974) and they have identical electrophoretic mobilities (Hörtnagl et al., 1972; Foldes et al, 1972; Kuzuya and Nagatsu, 1972). Soluble DβH can also be purified by concanavilin A - sepharose affinity chromatography of the granule lysate (Aunis et al., 1975a Rush et al., 1974) and can be stained with carbohydrate stains in gels of the soluble proteins (Huber et al., 1979; Cahill and Morris, 1979; see chapter 4). Thus CBB stained band 1 of non-reducing gels of the soluble proteins would appear to be the soluble form of dopamine-β-hydroxylase. In my reducing gels of the soluble proteins DβH apparently co-migrates with chromogranin A, the major CBB staining component of the soluble proteins. The soluble form of DβH can, however, be located in reducing gels of the granule lysate by carbohydrate staining (see chapter 4).

The heterogeneous nature of DβH in reducing gels, as shown by CBB staining of granule membranes and DβH prepared by concanavilin A - sepharose affinity chromatography from the granule membrane and lysate and FITC-con A staining of membranes and lysate may reflect different degrees of glycosylation or that the subunits of DβH are not identical.

3.4.5 Identification of Chromomembrin B

Chromomembrin B is the name given to the second major staining area of gels of chromaffin granule membranes separated by SDS polyacrylamide gel electrophoresis (Winkler, 1971). It has a faster mobility than chromomembrin A (DβH) and has a molecular weight of 28,000 daltons (Bartlett and Smith, see Winkler, 1976). In 8% and 10% acrylamide gels of reduced granule membranes no obvious candidate for chromomembrin B can be identified since there are no major staining components with molecular weights in the 28,000 dalton region. Kirshner (1974) also failed to identify chromomembrin B in 10% acrylamide gels of reduced granule membranes. However, in gradient gels a second intensely staining component of reduced granule membranes is seen. Closer observation reveals that this band is a doublet. Bartlett and Smith (1974) have reported that when chromomembrin B is electrophoresized in high acrylamide concentrations it appears as a doublet. The mobility of this doublet in acrylamide gradient gels indicates molecular weights of 20,000 and 22,000 daltons and from their position in the polypeptide profile would appear to be CBB stained bands 49, 50 and 51 identified in 8% acrylamide gels of reduced granule membranes. These bands have molecular weights of 23,000,21,000 and 20,000 daltons (calculated from their mobilities in 8% acrylamide gels) and are poorly stained. However, staining of chromomembrin B has been observed to be variable (Bartlett and Smith, 1974). CBB stained bands 49, 50 and 51, like chromomembrin B, have the same mobilities in reducing and non-reducing gels.

3.4.6 Identification of Other Bands

CBB stained bands 28, 29 and 44 of gels of reduced chromaffin granule membranes have been identified as subunits of the Mg^{++} ATPase of this membrane by comparison of the gel patterns of the partially purified enzyme and the granule membrane. Actin is a very minor component of the granule membrane (Phillips and Slater, 1975, Jockusch et al., 1977) and is tentatively identified on the basis of its molecular weight as band 35. A different protein of similar molecular weight known as synaptin (Bock and Helle, 1977) probably also migrates in this region of gels of the reduced membranes.

Cytochrome b-561 of the granule membranes has been purified and has a reported subunit molecular weight of 4,380 daltons (Silsand and Flatmark, 1974). I can, however, only identify polypeptides of molecular weight greater than 10,000 daltons in gels of granule membranes. The presence of lipid obscures smaller components.

3.4.7 Separation of the Soluble Proteins of Chromaffin Granules by SDS Polyacrylamide Gel Electrophoresis.

29 CBB staining bands can be seen in acrylamide gradient gels of chromaffin granule lysate separated by SDS polyacrylamide gel electrophoresis under reducing conditions. The majority of these bands are low molecular weight, minor staining components whilst the major staining components characteristically migrate as broad bands. Other workers (Winkler et al., 1970; Bartlett and Smith, 1974; Kirshner, 1974), with the exception of Cahill and Morris (1979), observe few components in SDS gels of the granule lysate. However, all these profiles contain broadly staining components similar to those described here. The reason why I have identified more bands is probably due to better resolution and the use of acrylamide gradient gels capable of resolving very low molecular weight components (see earlier).

Chromogranin A is the major staining component of gels of the granule lysate by definition (Schneider et al., 1967). I find that chromogranin A comprises 20 - 35% of the stainable material in gels of the lysate which is less than previously published values which fall in the range 40 - 50% (Schneider et al., 1967; Smith and Winkler, 1967b; Smith and Kirshner, 1967). The molecular weight of chromogranin A is reported to be in the range 74,000 - 81,2000 daltons (Smith and Kirshner, 1967; Smith and Winkler, 1967b; Kirshner and Kirshner, 1969; Helle, 1971b). The mobility of chromogranin A in acrylamide gradient gels indicates a molecular weight of 66,000 daltons whilst its mobility in 8% acrylamide gel suggests one of 69,000 daltons. Kirshner (1974) has also calculated the molecular weight of chromogranin A from its mobility in gels following SDS gel electrophoresis and obtains a figure of 65,000 daltons. Thus it may be that chromogranin A migrates anomalously or may be partially degraded by contaminating proteases (see Winkler, 1976). It has been suggested that the other chromogranins are proteolytic fragments of chromogranin A. The mobility of chromogranin A is unaffected by reducing agents.

3.4.8. Cholesterol Content of Chromaffin Granule Membranes

Two groups have obtained values for the cholesterol content of chromaffin granule membranes which are in good agreement with one another. Winkler et al. (1970) found 0.64 mg cholesterol per mg protein while Schneider (1972) obtained a value of 0.63 mg cholesterol/mg protein. These values are both much higher than the figure of 0.23 mg cholesterol/mg protein achieved by Da Prada et al. (1972b) but are lower than the value of 0.94 mg cholesterol/mg protein that I obtained. The use of cholesterol oxidase to assay cholesterol may be responsible for some or all of the differences between my value and those of Winkler et al. (1970) and Schneider (1972).

3.4.9 Enzyme Activities of the Chromaffin Granule Membrane

3.4.9.1 Dopamine-β-Hydroxylase

Direct comparison between values in the literature for the activity of dopamine-β-hydroxylase (DβH) in the granule membrane is difficult because of the different assay conditions used. In particular various non-saturating substrate concentrations have been used especially when radioactive assay methods have been used. Thus Helle (1971[a]) reported an activity of 1675 nmol/min/mg protein using a substrate concentration of 10 mM whilst Winkler et al. (1970) obtained a value of only 0.2 nmol/min/mg protein when using 0.005 mM substrate. With a substrate concentration of 0.22 mM I have found a value of 11.1 nmol tyramine converted/min/mg membrane protein for the specific activitiy of DβH in granule membranes. The nearest values in the literature are 2.7 nmol substrate/min/mg protein from Phillips (1973) who used 0.023 mM tyramine and 3.5 nmol substrate/min/mg protein from Schneider (1972) using 0.57 mM tyramine.

DβH is known to lose activity during its purification. This loss can be prevented by including catalase during the purification procedure. Activity is also lost when granules are subjected to density gradient centrifugation. My results indicate that DβH may be unstable to freeze-thawing and/or storage at -20°C. For this reason granules were not freeze-thawed, as recommended by Winkler el al. (1970) in order to lyse them prior to membrane preparation. This may explain the low specific activities of DβH in the membrane reported by Winkler et al. (1970). The specific activities of DβH of granule membrane preparations is probably also dependent on the amount of soluble DβH adsorbed to the membrane.

3.4.9.2 Mg^{++} ATPase

Values for the specific activity of the Mg^{++} ATPase in chromaffin granule membranes are also extremely variable and have led to suggestions that this enzyme is also relatively unstable (Winkler, 1976). Schneider (1972) and Winkler et al. (1970) have reported similar values (47 nmol substrate converted/min/mg protein and 30 nmol/min/mg protein respectively) to that reported here (48.5 nmol/min/mg protein) although much higher values have been obtained (e.g. 282 Muller and Kirshner, 1975; see also Banks, 1965; Taugner, 1971a; Taugner and Wahler, 1974; Pollard et al., 1973). Banks (1965) and Taugner (1971b) found no loss in ATPase activity when granule membranes were prepared from granules. Contamination with mitochondrial membranes which have much higher ATPase activities may be responsible for some of the higher figures. Apps and Glover (1978) have purified the Mg^{++} ATPase of the granule membrane and shown it to be different from the mitochondrial ATPase. They obtained a specific activity of 127 nmol/min at 25°C with 2 mM Mg^{++} and at pH 8.3 for the granule membrane ATPase. The method of purifying membranes that I have used is very similar to that employed by Winkler et al. (1970), except that granules were sedimented down 1.8 M sucrose and the purified granules were not lysed by freeze-thawing, and Schneider (1972) who employed a final sucrose gradient step to further purify his membranes.

3.4.9.3 NADH:Acceptor Oxidoreductase

Only Flatmark's group have reported activities for the NADH:ferricyanide oxidoreductase activity of granules and granule membranes. Values for dialyzed granules were found in the range 0.06 - 0.32 nmoles NADH reduced/min/mg protein (Terland and Flatmark, 1973) which corresponds to activities of between 0.3 and 0.6 nmoles/min/mg protein for the granule

membranes assuming all this activity is located in the membrane and that one fifth of the total granule protein is found in the membrane. Flatmark et al. (1971) have also assayed the NADH:ferricyanide oxidoreductase activity of the granule membranes directly and obtained a value of 27 nmol/min/mg protein which although considerably higher than the earlier estimates is lower than the figure of 0.12 μmol/min/mg protein that I have obtained. If this activity is calculated from the data in Fig 4 of Flatmark et al. (1971) then a much higher (0.22 μmol/min /mg protein) estimate is obtained than that quoted in Table II and in the text and is in relatively good agreement with the specific activity of NADH:ferricyanide oxidoreductase that I have found. The assay conditions that I have used are identical to those used by Flatmark et al. (1971).

3.4.9.4 Phosphatidylinositol kinase

For the specifc activity of phosphatidylinositol kinase in chromaffin granule membranes I have obtained a value of 0.46 nmol P_i incorporated/min/mg protein at 37°C. This activity is lower than those obtained by other workers, Phillips (1973) reporting a value of 1.25 nmol/min/mg, Lefebvre et al. (1976) 4.4 and Muller and Kirshner (1975) 2.9. Only Trifaro's (1972) value is lower (0.13 nmol/min/mg) although the figure of 0.68 nmol/min/mg membrane protein calculated from the specific activity of phosphatidylinositol kinase in granules published by Phillips (1973), assuming 1/5th of the granule protein is in the membrane, is near to the activity I obtained.

3.4.9.5 Reduced v Oxidized Absorption Spectrum of Chromaffin Granule Membranes

Absorption spectra of granules and granule membranes have been previously reported (Spiro and Ball, 1958 and 1961; Banks, 1965; Ichikawa and Yamano, 1965; Flatmark et al., 1971; Silsand and Flatmark, 1974).

Absorption maxima have been reported at (between) 428 and 430 nm, 520 - 532 nm and 538 - 561 nm for the reduced membranes. These have been interpreted as indicating the presence of a b type cytochrome (b_{561}) in the granule membranes. Flatmark et al. (1971) have suggested the broad negative peak at 445 nm is due to a flavoprotein. I also find absorption maxima in the regions indicated above for dithionite reduced membranes but only ocassionally is a broad negative peak seen in the 445 nm region. I have tried to quantitate the amount of cytochrome b_{561} present in the membranes by measuring the difference in absorbance at 561 nm and 540 nm of the reduced versus oxidized difference spectrum of granule membranes and obtained a figure of 0.11 absorbance units/mg membrane protein. From Fig. 4 of Flatmark et al. (1971) a value of 0.162 absorbance units/mg protein for granule membranes can be calculated for this parameter as can the value 0.103 absorbance units/mg nitrogen (assuming 4/5th of the large granule protein is soluble and that all the cytochrome b_{561} is present in the membrane and using the value of 4.4 mg N/g medulla from Table I) from Fig 2 of Spiro and Ball (1961). Insufficient data (usually absence of the protein concentration) prevents comparisons between the amount of cytochrome b_{561} in the granule membranes of other workers who have published absorption spectra of reduced granule membranes.

CHAPTER 4

IDENTIFICATION AND TOPOGRAPHY OF GLYCOPROTEINS OF THE CHROMAFFIN GRANULE MEMBRANE

IDENTIFICATION AND TOPOGRAPHY OF GLYCOPROTEINS OF THE CHROMAFFIN GRANULE MEMBRANE

4.1 Introduction

Glycoproteins of plasma membranes have been implicated in a number of cell surface recognition phenomena (Hughes, 1973) and it has been suggested that glycoproteins of the chromaffin granule membrane play a similar role in the recognition and initial contact phases of exocytosis (Meyer and Burger, 1976). Preliminary reports on the topography of glycoproteins in the granule membrane (Meyer and Burger, 1976; Eagles et al., 1975) indicate that the glycoproteins may be exposed at both surfaces of the membrane. This would make the granule membrane unique since work on other membranes has shown that carbohydrate is only exposed at one surface of a particular membrane - the outer surface of plasma membranes (Nicolson and Singer, 1974) and the inner surface of intracellular membranes (Virtanen and Wartiovaara, 1976; Hirano et al., 1972: Lewis et al., 1977). Therefore identification and confirmation of the localization of glycoproteins of the chromaffin granule membrane is of considerable interest.

Several staining techniques exist for detecting glycoproteins in polyacrylamide gels, the most common of which is the periodic acid - Schiff stain. After fixation of the proteins and elution of sodium dodecyl sulphate, the gels are oxidized with periodate and stained with the Schiff reagent. A pink coloured compound is formed between the Schiff reagent and the oxidized sugar residues of the glycoprotein. Since sialic acid residues are the most easily oxidized by periodate (Steck, 1974), the periodic acid - Schiff stain is most sensitive towards sialoglycoproteins. Indeed certain glycoproteins which do not contain sialic acid may not be stained at all by this method.

Eckhardt et al. (1976) have used the fluorescent reagent 1-dimethylaminonapthalene-5-sulphonylhydrazine (dansyl hydrazine) to stain glycoproteins in gels. Aldehyde groups of sugar residues generated by periodate oxidation condense with dansyl hydrazine to form hydrazones which are reduced to stable hydrazine derivatives with sodium borohydride. The fluorescently labelled glycoproteins can then be visualized with long wavelength ultraviolet light. The dansyl hydrazine stain is more sensitive than the periodic acid - Schiff stain but requires more sophisticated instrumentation (a fluorescence gel scanner) to record and quantitate the stain. Because of its dependence upon periodate oxidation, the dansyl hydrazine stain is also most sensitive in detecting sialoglycoproteins.

Two other glycoprotein stains have been reported in the literature, viz: , "Stains-all" (1-ethyl-2-[3-(1-ethylnapthol [1,2d]thiazolin-2-ylidene)-2-methylpropenyl]-napthol 1, 2d-thiazolium bromide) and the hydrazinoacridine stain. Both these methods simultaneously detect glycoproteins and non-glycoproteins. After staining with "Stains-all" sialoglycoproteins appear blue, non-glycoproteins red and lipid yellow-orange (King and Morrison, 1976; Green and Pastewka, 1975). However, other acidic macromolecules (such as nucleic acids, phosphoproteins and glycosaminoglycans) also stain blue. Therefore only components which stain blue before and red purple after neuraminidase digestion (Green and Pastewka, 1975) can be identified positively as sialoglycoproteins using "Stains-all". Treatment of periodate oxidized gels with hydrazinoacridine (Carson, 1977) results in glycoproteins fluorescing yellow when illuminated with long wavelength ultraviolet light. Non-glycoproteins also stain fluorescently but their fluorescence is less intense and a different colour to that of glycoproteins.

Perhaps the most powerful technique for detecting glycoproteins in polyacrylamide gels involves the use of lectins. Lectins are proteins containing specific binding sites for sugars (see Lis and Sharon, 1973 for review) and will bind to glycoproteins containing the specific hapten sugar(s) of the lectin. Many lectins are known with different sugar specificities and, therefore, the use of several different lectins with different sugar specificities should allow most types of glycoproteins to be identified. Lectins used for gel staining and the glycoproteins to which they bind can be visualized by labelling the lectins with fluorescein (West and McMahon, 1977; Ash and Singer, 1976; Nicolson, 1973) or ^{125}I (Gurd, 1977a;Rostas et al., 1977; Burridge, 1976; Tanner and Anstee, 1976; Robinson et al., 1975). A variation of the lectin staining technique is the use of lectin affinity chromatography (Gurd, 1977a; Gurd and Mahler, 1974; Zanetta et al., 1975; Kahane et al., 1976; Lotan et al., 1977). Glycoproteins bound to the lectin affinity column are removed by the specific hapten sugar of the lectin and the eluted material is then subjected to polyacrylamide gel electrophoresis.

Methods commonly used to discover which side of the membrane glycoproteins are exposed at include tritiated borohydride treatment of galactose oxidase or periodate treated intact cells or organelles and digestion with proteases or neuraminidase. After treatment of intact cells or organelles with one of the methods listed above membranes are prepared and subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. In the case of the galactose oxidase or periodate techniques tritium labelled glycoproteins can be located in the gel by fluorography or by liquid scintillation counting of digested gel slices (Trishler and Epstein, 1968; Cain and Pitney, 1968; Zaitlin and Hariharasubramanian, 1970; Basch, 1968). Protease or neuraminidase digested glycoproteins, stained with coomassie brilliant blue or

one of the glycoprotein stains, should show altered mobilities compared to their undigested forms.

Galactose oxidase from Polyporous circinatus (Avigad et al., 1962) is available as a partially purified preparation. Its use as a vectorial labelling reagent is due to its inability to penetrate membranes because of its size. Tritiated borohydride can freely traverse membranes but will only reduce galactose residues of glycoproteins or glycolipids which have been oxidized by galactose oxidase. Thus Steck and Dawson, (1974) have used this technique to investigate the topographical distribution of carbohydrate in the erythrocyte plasma membrane whilst Gahmberg and Hakomori (1973a) studied the effect of protease and neuraminidase treatment on the tritiated borohydride labelling pattern of plasma membranes of galactose oxidase treated intact normal adult and foetal erythrocytes. Changes in the external labelling pattern of glycoprotein and glycolipid of cell membranes caused by transformation (Gahmberg and Hakomori, 1973b) and by different stages of the cell cycle (Gahmberg and Hakomori, 1974) have also been followed. By treatment of isolated membranes with galactose oxidase and tritiated borohydride this method can also be used to detect galactose containing glycoproteins of the membrane.

Gahmberg and Andersson (1977) have shown that treatment of intact cells with low concentrations of periodate at $0^{o}C$ results in oxidation of externally located glycoproteins only. Under these conditions sialic acid residues in the glycoprotein are selectively oxidized. Thus periodate oxidation followed by reduction with tritiated borohydride has been used to detect externally located sialoglycoproteins of the plasma membranes of erythrocytes (Liao et al., 1973; Mueller et al., 1976), erythrocyte variants (Gahmberg et al., 1976a) and other cells (Gahmberg et al., 1976b; Critchley et al., 1976). Treatment of isolated membranes will identify all the sialoglycoproteins of the membranes.

Problems associated with protease digestion of intact cells or organelles are fully discussed in chapter 5. If a particular glycoprotein is missing from its normal position in the coomassie brilliant blue or glycoprotein staining profile of a membrane following protease digestion of the intact structure and sodium dodecyl sulphate polyacrylamide gel electrophoresis then it is likely that at least a part of the glycoprotein is externally located in the membrane. It is much more difficult to decide whether the carbohydrate moiety of the glycoprotein is on the outside of the cell or organelle. This requires the location of the digested product of the glycoprotein which has remained attached to the membrane in the staining profile of the SDS gel of the digested membrane (which may be difficult if extensive digestion has taken place and many new bands have arisen) to see if the carbohdyrate moiety is still attached. Easier, perhaps, is to analyse the supernatant of digested cell or organelle incubation media for carbohydrate.

In this chapter the identification of glycoproteins in gels of chromaffin granule membranes is reported. The periodic acid - Schiff and dansyl hydrazine stains, which are sensitive mainly to sialoglycoproteins, have been used as well as two fluorescein conjugated lectins, concanavilin A and wheat germ agglutinin both of which have been shown to bind to chromaffin granule membranes (Eagles et al., 1975; Meyer and Burger, 1976). Concanavilin A, from Canavalia ensiformis binds to D-mannose and D-glucose and glycoproteins containing these sugar residues whilst wheat germ agglutinin specifically interacts with N-acetylglucosamine (Lis and Sharon, 1973). For comparison gels containing the soluble proteins of the chromaffin granule have also been stained for carbohydrate using these methods. The sidedness of the carbohydrate moieties of these glycoproteins in the granule membrane has been investigated using the

galactose oxidase/tritiated borohydride and periodate/tritiated borohydride techniques. The effect of protease digestion of intact granules and isolated granule membranes on the periodic acid - Schiff staining profile of gels of the membranes is reported elsewhere (see chapter 5).

4.2. Materials and Methods

4.2.1. Materials

Concanavilin A and wheat germ agglutinin derivatized with fluorescein isothiocyanate (FITC-ConA and FITC-WGA respectively) were gifts from Dr. A. Cahill of the Max-Planck-Institut für biophysikalische Chemie, Gottingen, Federal Republic of Germany (see Cahill and Morris, 1979 for preparation of FITC-ConA and FITC-WGA). Basic fuschin was donated by Dr. I. Sweetin of this department. Dansyl hydrazine and Galactose oxidase (Sigma Type II from Polyporous circinatus, a partially purified powder containing 70% sucrose, activity 50 - 100 units/mg solid, one unit producing a change in absorbance at 425 nm of 1.0 per minute at pH 6 at 25^{o}C in a Peroxidase and O-Tolidine system of reaction volume 3.4 ml and using a light path of 1 cm) were obtained from Sigma and [^{3}H]potassium borohydride (50 mCi/mmole) from the Radiochemical Centre, Amersham, England.

4.2.2. The Periodic Acid - Schiff stain (PAS stain)

The Schiff stain was prepared according to the method of Fairbanks et al. (1971). After electrophoresis gels were either stained with coomassie brilliant blue or the PAS stain according to Fairbanks et al (1971). The PAS stained gels were photographed using Kodalith film Type III, deep orange and green filters, and an exposure time of between two and three minutes and also densitometrically scanned at 560 nm with a Gilford spectrophotometer. Control gels, in which the periodate oxidation step was omitted, were also stained with Schiff reagent.

4.2.3. The Dansyl Hydrazine Stain

The method of Eckhardt et al. (1976) was adopted and the stained gel scanned for fluorescence using the modified Gilford linear transport spectrophotometer (see section 2.2.3). Controls, in which non-oxidized gels were stained with dansyl hydrazine, were again performed. After fluorescence scanning, dansyl hydrazine stained gels were also stained with coomassie brilliant blue (see section 2.2.1).

4.2.4 Fluorescein-Labelled Lectin Staining

Gels were stained with FITC-lectin using a modification of the method of Rostas et al. (1977). After fixation with 25% (v/v) isopropanol, 10% (v/v) acetic acid (1 - 2 days) and equilibration in phosphate buffered saline, the gels were placed in a shallow dish and covered with a small amount (10-15 ml) of either FITC-ConA or FITC-WGA (0.1 - 0.2 mg/ml) in phosphate bufferred saline. A glass lid was placed on top and the whole agitated on a shaking table for one hour at room temperature. Destaining was performed with phosphate bufferred saline. FITC-lectins were stored frozen and re-used several times. Fluorescence scanning of FITC-lectin stained gels was performed with the modified fluorescence microscope fitted with a motorized stage,belonging to and built by Dr. S.J. Morris of the Max-Planck-Institutefur biophysikalische Chemie, D-3400 Gottingen-Nikolausberg, Bundesrepublik Deutschland and described in Cahill and Morris (1979).

The specificity of staining with FITC-lectins was determined by destaining the FITC-lectin stained gels in the presence of the hapten sugar (0.2 M in PBS) of the lectin (D-mannose for Con A and N-acetyl-glucosamine for WGA). Gels previously stained with FITC-lectin could be stained with coomassie brilliant blue following equilibration in 5:5:1 methanol:water:acetic acid.

4.2.5 Galactose Oxidase/[^{3}H]Potassium Borohydride Treatment

The general procedure of Gahmberg(1976) and Gahmberg and Hakomori (1973a) was followed. P_2 granules in 0.3 M sucrose, 10 mM hepes, pH 7 (1 ml, 10 mgs protein per ml) were incubated at room temperature for three hours with thirty-four units of galactose oxidase. After incubation the granules were washed with 5 mls 0.3 M sucrose, 10 mM hepes, pH 7, the granules being recovered by centrifugation at 18,000gav for 20 min at 4°C. Solid [^{3}H]potassium borohydride (approx 250 μCi) was added to the granules resuspended in 0.5 ml bufferred sucrose which were then left at room temperature for 30 min. Excess cold sodium borohydride (solid) was added and the granules left at room temperature for one hour. The granules were purified in the normal manner and granule membranes prepared from them (see section 2.1.2).

Purified chromaffin granule membranes (0.5 ml, 2 mg/ml protein) were labelled in a similar manner. After incubation with 17 units galactose oxidase (for 3 hr at R.T.), the membranes were washed twice with 10 mls 10 mM hepes, pH 7 and by centrifugation at 160,000 gav for 1 hr at 2°C. The membranes, in 0.5 ml 10 mM hepes, pH 7, were reduced with 250 μCi potassium borohydride (30 min, R.T.), incubated with cold sodium borohydride for 1 hr and then washed twice with 10 ml 10 mM hepes, pH 7 and by centrifugation at 160,000 g-av for 1 hr at 2°C.

4.2.6 Periodate/[^{3}H]potassium borohydride treatment

The labelling conditions were based on those of Gahmberg and Andersson (1977). P_2 granules (1 ml in bufferred sucrose, 10 mg/ml protein) were incubated with 1 mM sodium periodate for 10 min at 0°C. The reaction was stopped with 0.2 ml 0.1 M glycerol in 0.3 M sucrose, 10 mM hepes, pH 7 and the oxidized granules then washed, resuspended, reduced with [^{3}H]borohydride, purified and converted to granule membranes as described above for galactose oxidase/[^{3}H]potassium borohydride treatment.

Granule membranes (0.5 ml in 10 mM hepes, pH 7 and 2 mg per ml protein) were oxidized with periodate under the same conditions as those used for granules. Washing, reduction with [^{3}H]potassium borohydride, and further washing of the membranes was performed as described for the treatment of purified granule membranes with galactose oxidase/ [^{3}H]potassium borohydride.

4.2.7 Counting Tritium in Polyacrylamide Gels

Tritiated membrane samples were subjected to SDS polyacrylamide gel electrophoresis and the gel stained with coomassie brilliant blue and densitometrically scanned at 570 nm. Using the apparatus previously described (see section 6.2.7) the gel was sectioned into 1 mm slices. These were 'solubilized' by heating at 50^{o}C in 1 ml 9 parts NCS to 1 part water for approximately 2 hrs in capped vials. At the end of this period the gel slices had swollen to almost three times their original size and 2.5 ml toluene based scintillation fluor was added when the samples had cooled to room temperature. Counting was performed in a Searle liquid scintillation spectrometer. Individual counting efficiencies were computed and converted to dpm by the counter.

4.2.8 Other Methods

SDS polyacrylamide gel electrophoresis, preparation of granules, granule membranes and granule lysate and protein determination were performed as described in chapter 2.

4.3 Results

4.3.1. The Periodic Acid - Schiff Stain

Gels of granule membranes separated by SDS polyacrylamide gel electrophoresis under reducing conditions were very poorly stained by the PAS procedure when amounts of protein (100 to 150 µgms) which give good separation and staining with coomassie brilliant blue were applied

to the gel. Up to double this amount of protein was needed in order to obtain a sufficient density of staining to allow the gel to be photographed or densitometrically scanned. This resulted in distortion and poor separation of protein bands if gels containing 200 - 300 µgms protein were stained for protein. Thus for comparison of the PAS staining profile of granule membranes with the CBB staining profile, slab gels containing duplicate lanes of several protein loadings, one of each loading being stained with the PAS stain and the other with CBB, were run.

On 8% acrylamide gels of reduced granule membranes 5 PAS bands (excluding staining at the top of the gel, the dye front and lipid region) can be seen (Fig. 4.1). Use of 10% acrylamide and 8% - 20% acrylamide gradient gels, which are able to resolve smaller molecular weight components than the 8% acrylamide gels, fail to detect any PAS staining bands migrating ahead of PAS V (see Figs. 4.2 and 4.3).

PAS staining bands are much broader than coomassie brilliant blue staining bands. Thus several CBB bands (CBB bands 4 - 12 inclusive) migrate in the same region of the gel as PAS I. The CBB bands are only minor staining components and therefore it seems unlikely that they represent PAS I in the CBB staining profile. PAS II co-migrates with CBB bands 13 to 19 approximately. CBB bands 13, 14 and 15 have been identified as the reduced form of dopamine-β-hydroxylase (see section 3.4.4) and in view of the fact that DβH is a glycoprotein it seems likely that PAS II represents DβH. PAS III migrates with CBB bands 23 to 27 of the reduced granule membranes, PAS IV with CBB bands 28 to 35, and PAS V with CBB bands 36 - 40. Chromomembrin B previously identified as reduced CBB bands 49, 50 and 51 (see section 3.4.5) is not stained by the PAS procedure.

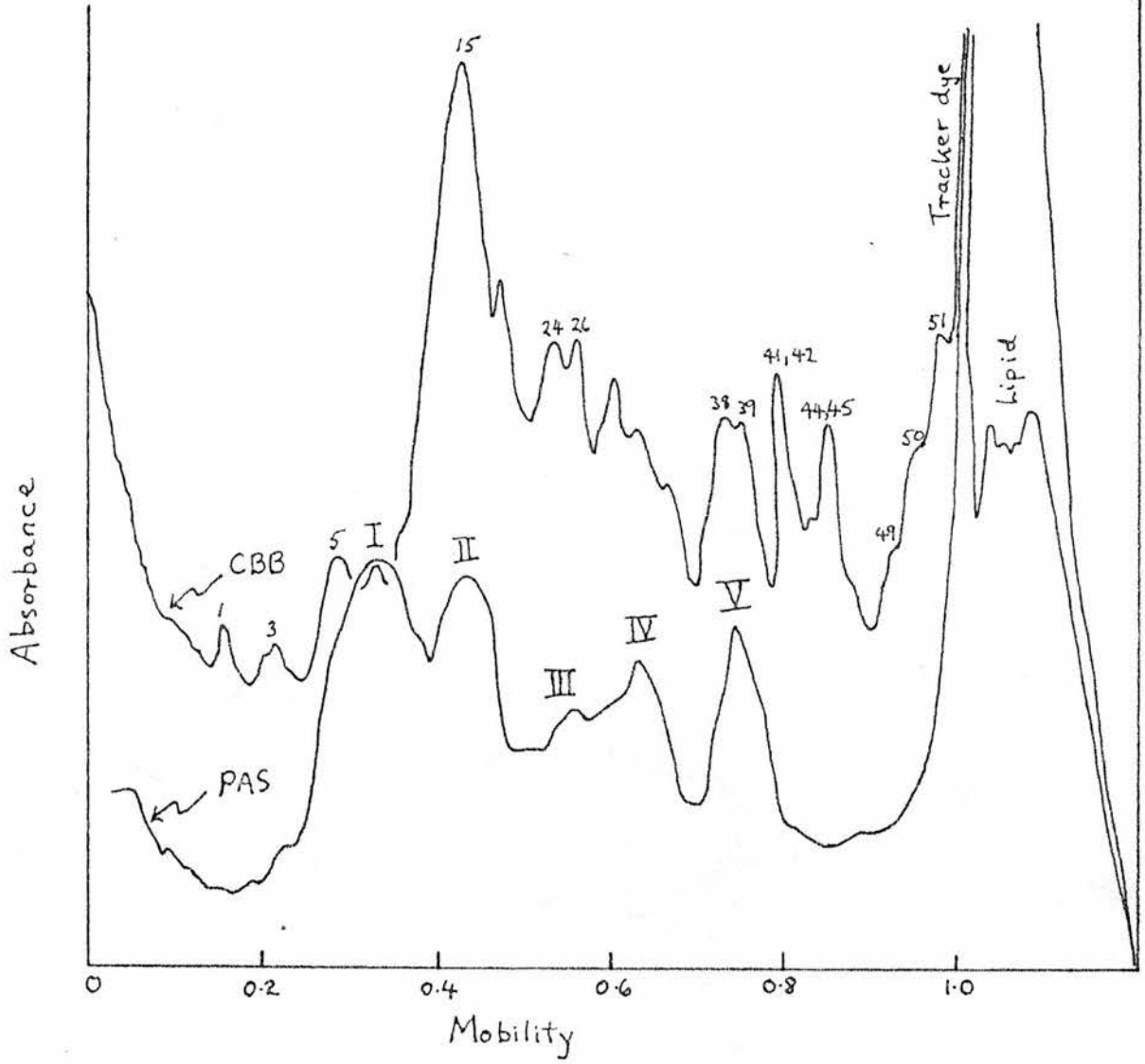


Fig. 4.1 The periodic acid - Schiff Staining Components of Reduced Chromaffin Granule Membranes

Chromaffin granule membranes (200 μgm protein) were separated by SDS polyacrylamide gel electrophoresis under reducing conditions in 8% (w/v) acrylamide gels. Following staining by the periodic acid - Schiff procedure (see section 4.2.2) the gel was densitometrically scanned at 560 nm. Also shown for comparison is the densitometric scan (570 nm) of an equivalent gel (100 μgm protein) stained for protein with coomassie brilliant blue.

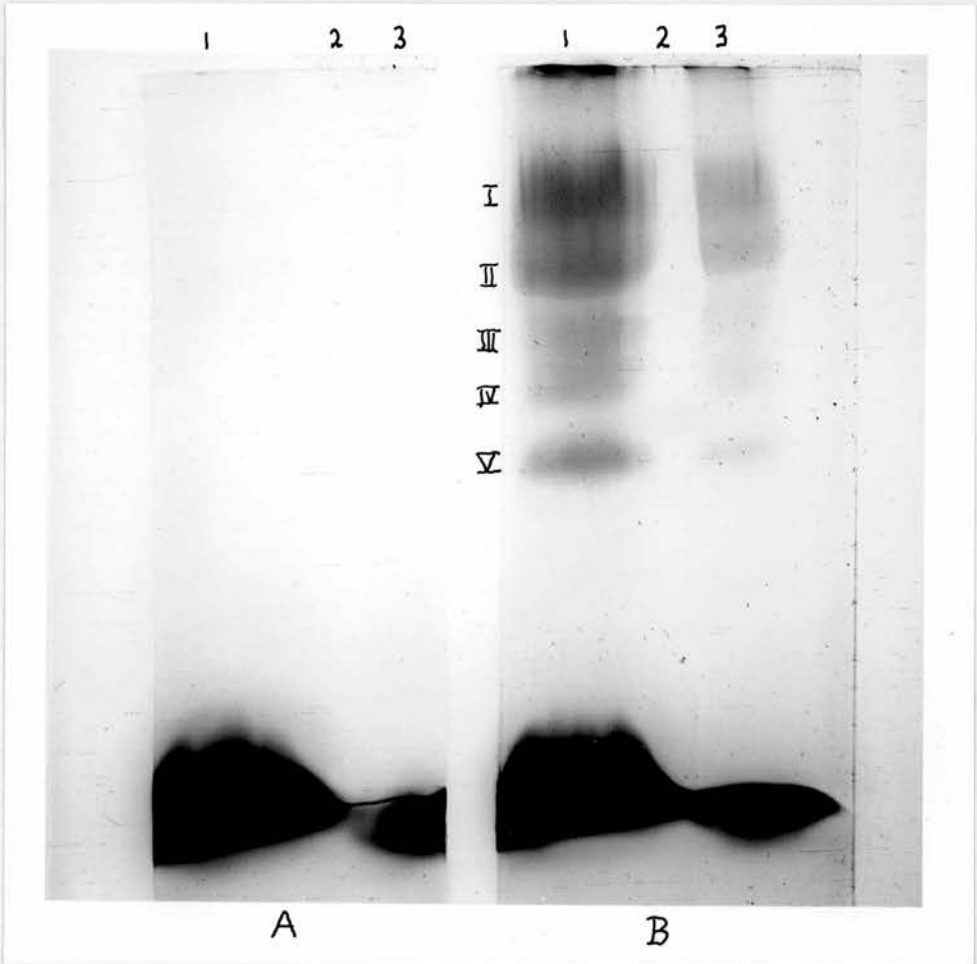


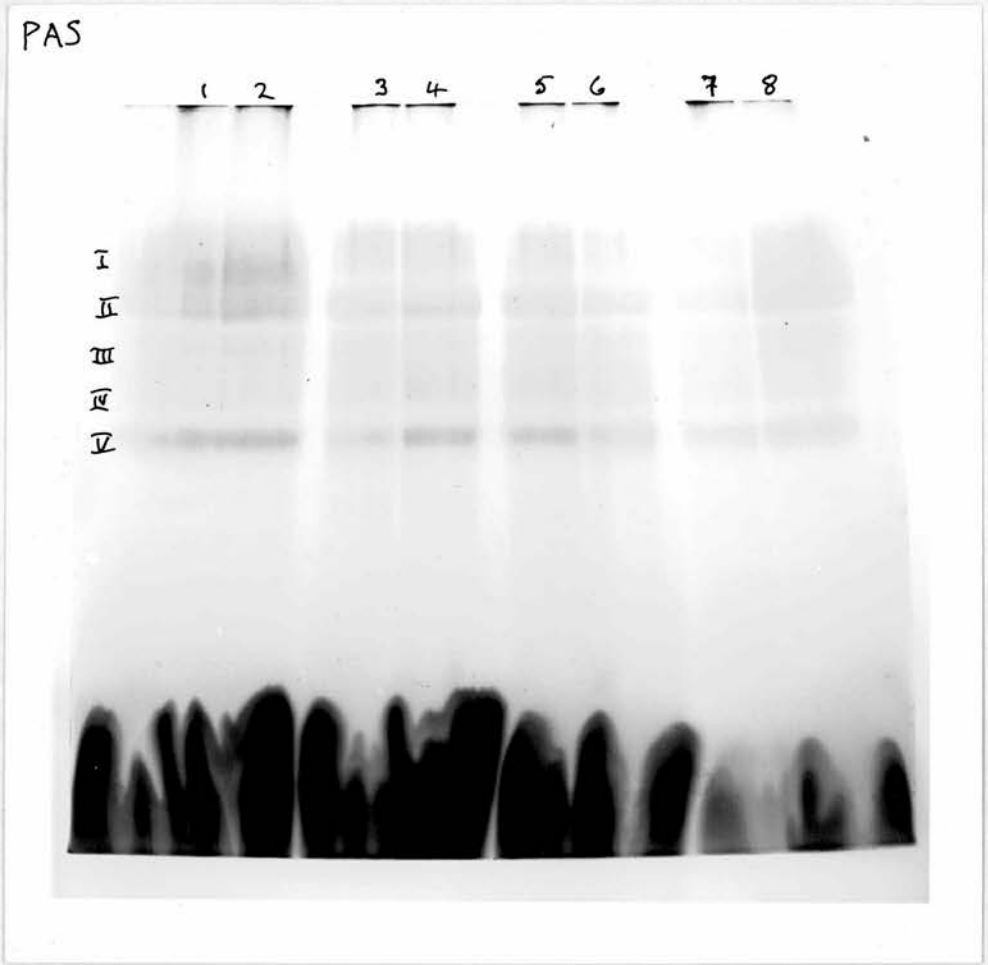
Fig 4.2 Staining with Schiff Reagent in the Absence of Periodate Oxidation

Gels (10% acrylamide) A and B are duplicate gels each containing in lane 1, chromaffin granule membranes, 250 µgm protein; lane 2, 50 µgm each bovine serum albumen and ovalbumen; lane 3, chromaffin granule membranes, 150 µgm protein. After SDS polyacrylamide gel electrophoresis under reducing conditions both gels were stained by the PAS procedure except that, in the case of gel A, the periodate oxidation stage was omitted.

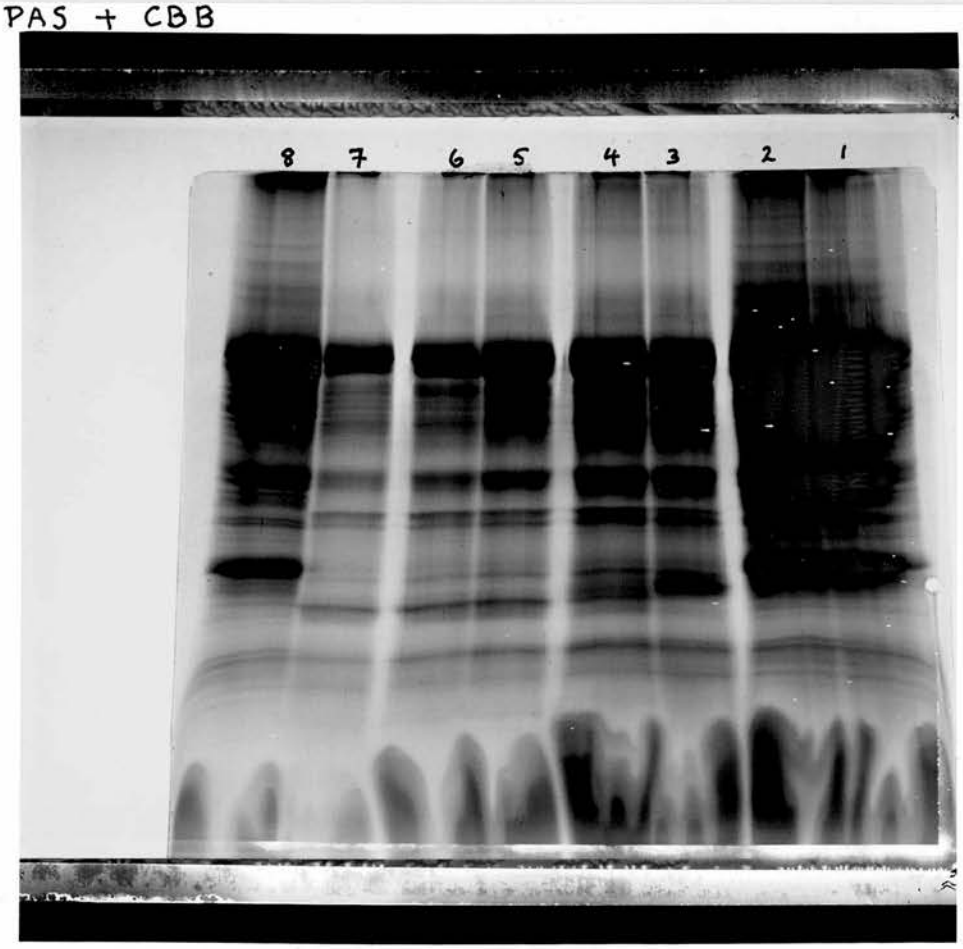
Fig. 4.3 Counter Staining of PAS Stained Gels of Chromaffin Granule Membranes with CBB

SDS polyacrylamide gel electrophoresis was performed in an 8-20% (w/v) acrylamide gradient gel under reducing conditions. The gel was initially stained by the PAS procedure and then counterstained with coomassie brilliant blue. Lanes 1, 2 and 8 contain chromaffin granule membranes (approx. 150 μgms protein/lane) whilst lanes 3-7 contain various granule membrane preparations digested with pronase (see chapter 5).

PAS



PAS + CBB



Gels stained by the PAS method in the absence of periodic acid treatment (Fig. 4.2) showed no staining in the protein region of the gel of reduced granule membranes. Initial staining of non-oxidized gels did reveal a similar PAS profile to that of oxidized gels but in the former case this staining was removed by destaining in sodium metabisulphite. However staining of the dye front and lipid region of non-oxidized gels remains after destaining. PAS staining of oxidized gels containing known glycoproteins and non-glycoproteins (Fig. 4.2) showed initial staining of all protein classes but after complete destaining only the glycoproteins remained stained. Thus, provided gels are destained completely (as indicated by the failure of the wash solution to turn pink upon the addition of a drop of formaldehyde) then the PAS stain is specific for glycoproteins.

PAS stained gels of chromaffin granule membranes were counter-stained for protein with coomassie brilliant blue (see Fig. 4.3). The CBB staining profile obtained was identical to that of gels containing granule membranes stained for protein with CBB immediately after electrophoresis. Thus the fixation process retains all the CBB staining polypeptides of the reduced granule membranes.

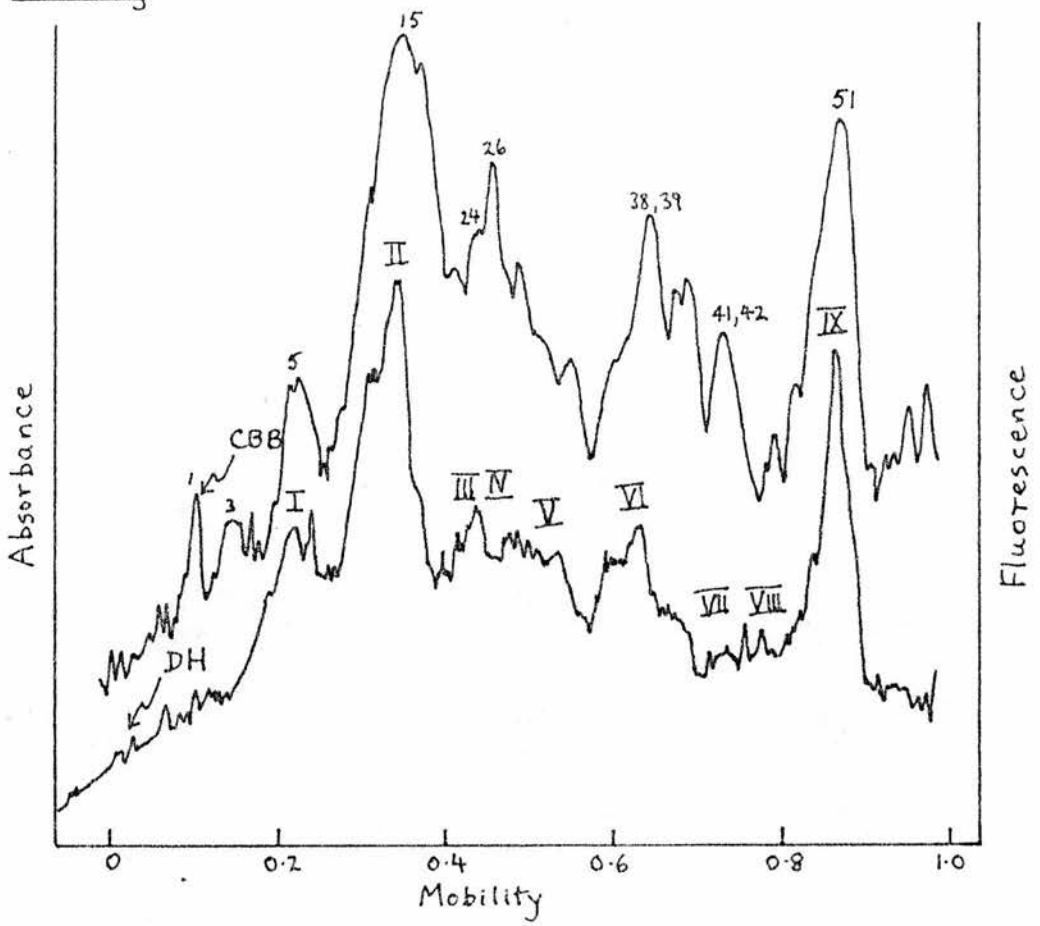
4.3.2 The Dansyl Hydrazine Stain

The dansyl hydrazine stain is much more sensitive than the PAS stain (see Eckhardt *et al.*, 1976) and therefore gels optimally loaded with protein can be used for glycoprotein staining by this technique. Gels (10% acrylamide) of reduced chromaffin granules separated by SDS polyacrylamide gel electrophoresis and stained with dansyl hydrazine according to the method of Eckhardt *et al.* (1976) revealed 9 bands (DH I to IX Fig. 4.4a) in addition to staining at the top of the gel, tracker dye and lipid region of the gel. On the basis of their

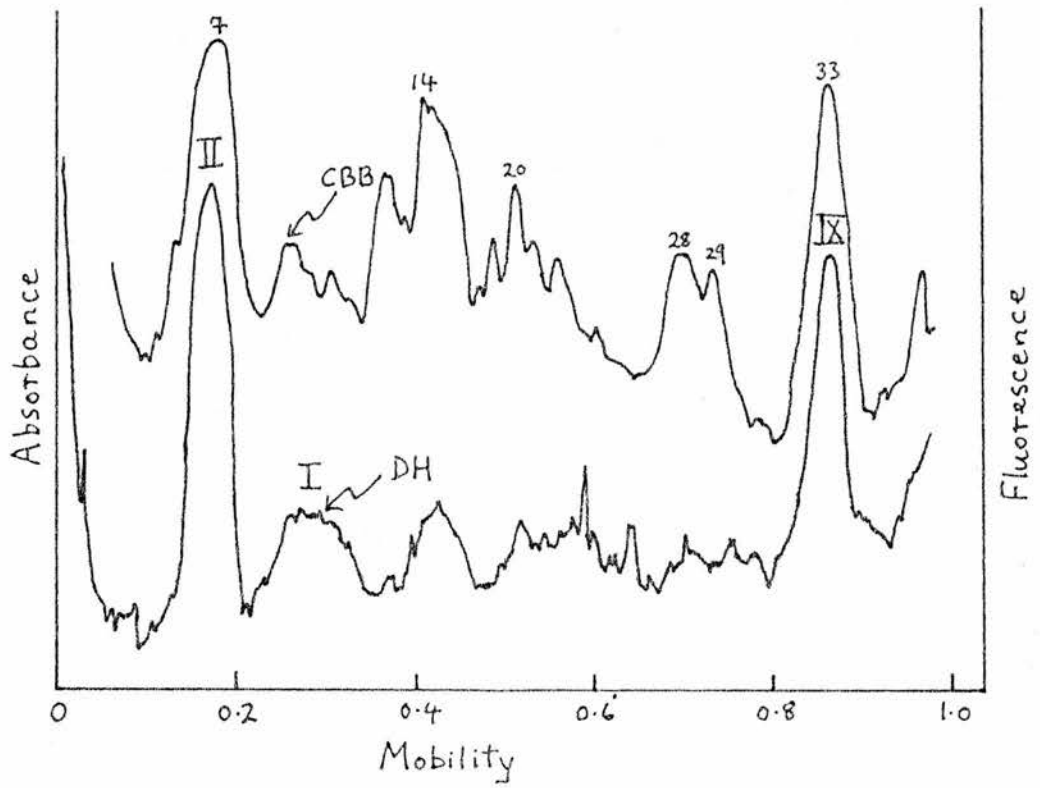
Fig. 4.4 Dansyl Hydrazine Binding Components of Reduced and Non-Reduced Chromaffin Granule Membranes

Granule membranes (approx. 100 μgms protein) were separated under reducing (a) or non-reducing (b) conditions of SDS polyacrylamide gel electrophoresis in 10% (w/v) acrylamide gels and then stained for carbohydrate by the dansyl hydrazine method (see section 4.2.3). The stain was detected by scanning the gel for fluorescence. The densitometric scans of equivalent gels stained for protein with coomassie brilliant blue are also shown.

a. Reducing



b. Non-reducing



GP	M. Wt. 10^{-3} daltons	CBB bands	PAS	DH	FITC-Con A	FITC-WGA	GO/ $[^3H]BH_4$	PER. $[^3H]BH_4$	Comments
I	103	4-12	I	I	I	I	I		Unaffected by DTT. Does not stain with CBB. 2 GPs?
II	73	13-19	II	II	II	II	II	I	DβH. Oxidized m.wt. c. 150,000 daltons
III	68	20-23					III		
IV	58	24,25	III	III	III	III			Possibly affected by DTT
V	56	26,27		IV	IV				Possibly affected by DTT
VI	50	28-35	IV	V	V		IV		Unaffected by DTT
VII	37	36-40	V	VI	VI	IV	V		Represents background CBB staining to bands 36-40
VIII	32	41-43		VII	VII		VI		
IX	28	44-46		VIII	VIII				
X	23	47,48			IX				

Table 4.1 Glycoproteins of the Chromaffin Granule Membrane

relative mobility compared to that of the CBB stained bands of the reduced granule membrane and their relative staining intensity most of the dansyl hydrazine bands could be identified with PAS bands (see Table 4.1). A major difference between the dansyl hydrazine and PAS profiles was the staining of chromomembrin B (CBB bands 49, 50 and 51; DH band IX) with dansyl hydrazine. Other differences in the profiles (DH band VII and VIII) were minor ones and could be attributed to the greater sensitivity of the dansyl hydrazine stain. Similarities between the dansyl hydrazine and PAS profiles are to be expected because of their dependence upon periodate oxidation prior to staining.

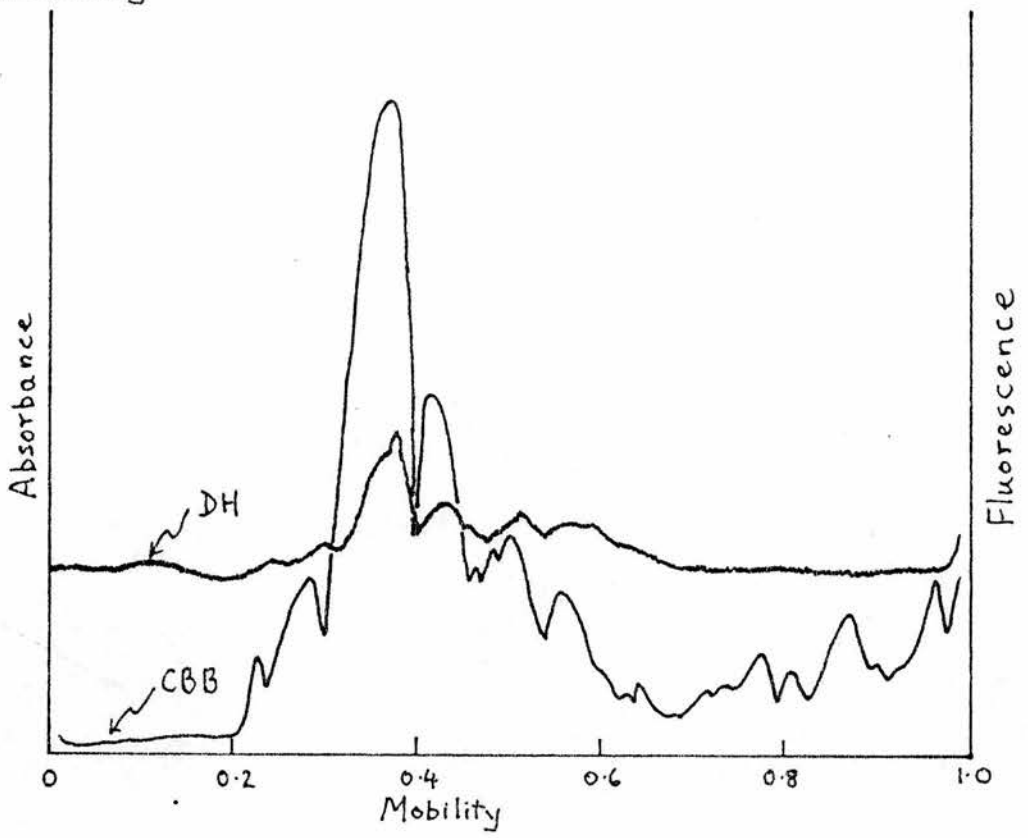
Dansyl hydrazine staining of gels of non-reduced chromaffin granule membranes (Fig. 4.4b) confirmed the staining of dopamine-β-hydroxylase and chromomembrin B in reduced gels. DβH exhibits a decreased mobility under non-reducing conditions of electrophoresis and the dansyl hydrazine peak identified as DβH (DH II in reducing gels) also shows this behaviour. The mobility of chromomembrin B is unaffected by reducing agents and DH IX remains in the same region of the gel under both conditions of electrophoresis. DH I and V are also unaffected by reducing agents but DH VI is clearly missing from the non-reduced DH profile of granule membranes. Only one new band appears in the non-reduced DH profile this banding having a mobility slightly faster than that of reduced DβH. It is possible that this band is the non-reduced form of DH VI.

For comparison, gels of the soluble proteins of the granule were also stained with dansyl hydrazine. Only one weakly fluorescent band was observed in both reduced and non-reduced gels (Fig. 4.5) and from its electrophoretic behaviour appears to be the soluble form of dopamine-β-hydroxylase. Very small broad fluorescent peaks were observed but never consistently and these have, therefore, not be catalogued.

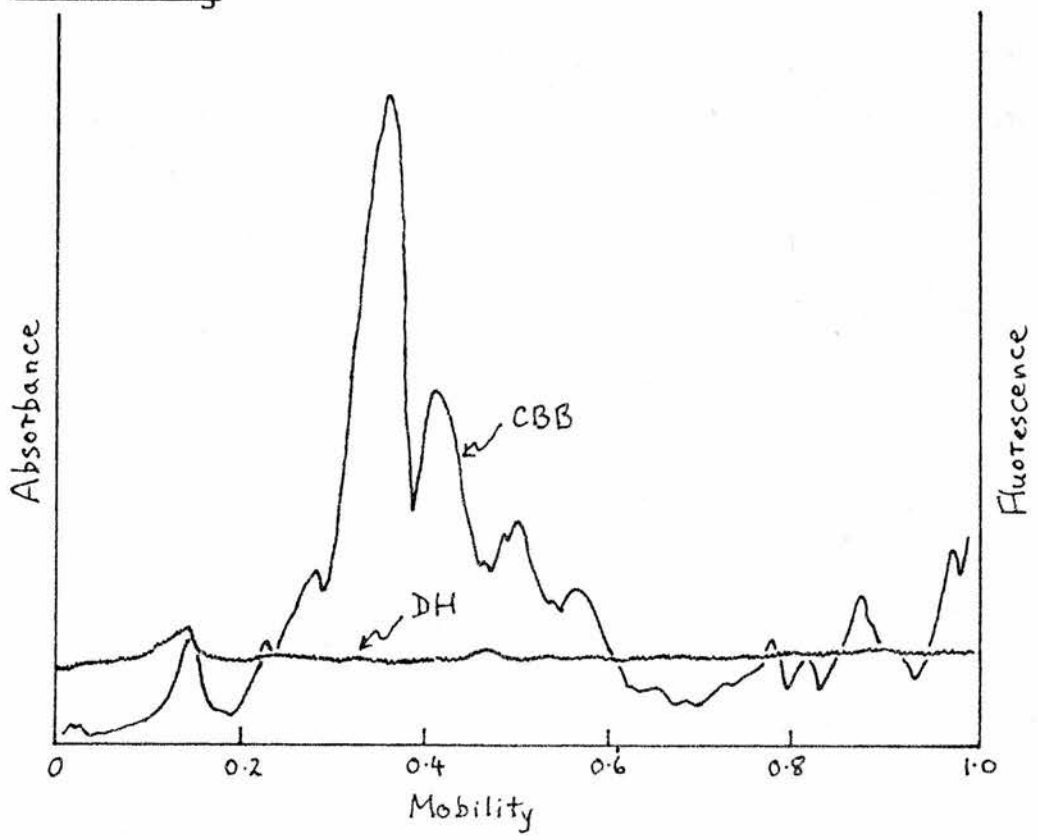
Fig. 4.5 Dansyl Hydrazine Binding Components of Reduced and Non-Reduced Chromaffin Granule Lysate

Gels (10% acrylamide) containing chromaffin granule lysate (approx. 100 μgm protein) separated under reducing (a) or non-reducing (b) conditions of SDS polyacrylamide gel electrophoresis were stained by the dansyl hydrazine procedure. Glycoproteins are indicated by peaks in the fluorescence profile of the gel. Also included are typical CBB staining profiles of gels of reduced and non-reduced chromaffin granule lysate.

a. Reducing



b. Non-reducing



Counter-staining with CBB of dansyl hydrazine treated gels containing the soluble proteins (Fig. 4.6) showed very poor staining of the majority of the components compared to their staining in equivalently loaded gels stained immediately after electrophoresis. This poor staining is unlikely to be due to the presence of dansyl hydrazine for two reasons. Firstly gels containing either reduced or non-reduced granule membranes which have previously been stained with dansyl hydrazine do not show impairment of staining with coomassie brilliant blue and they are more heavily stained with dansyl hydrazine than gels of the lysate proteins. Secondly in gels of the soluble proteins DβH is stained with coomassie brilliant blue to the same extent whether the gels are firstly stained with dansyl hydrazine or not and this component clearly reacts with dansyl hydrazine. It seems likely therefore, that the majority of the soluble proteins, with the exception of DβH, are eluted during the fixation and/or staining stages of the dansyl hydrazine procedure. Hence it is not surprising that so few fluorescent bands are observed when gels of the lysate proteins are stained with dansyl hydrazine.

A more serious problem concerned the ability of dansyl hydrazine to stain oxidized and non-oxidized gels equally well. Both oxidized and non-oxidized gels destained at the same rate and this was followed till stainable material in both gels could no longer be discerned. Non-oxidized gels were stained a slightly different shade of yellow-green fluorescence. Furthermore bovine serum albumen and ovalbumen were equally well stained in periodate oxidized gels and this may explain why chromomembrin B is stained with dansyl hydrazine but not by the PAS procedure. However, some degree of specificity is clearly present since not every polypeptide is stained in oxidized gels and there is fairly good agreement with the PAS stain for which the controls exhibited the expected behaviour. For these reasons the results of the dansyl hydrazine

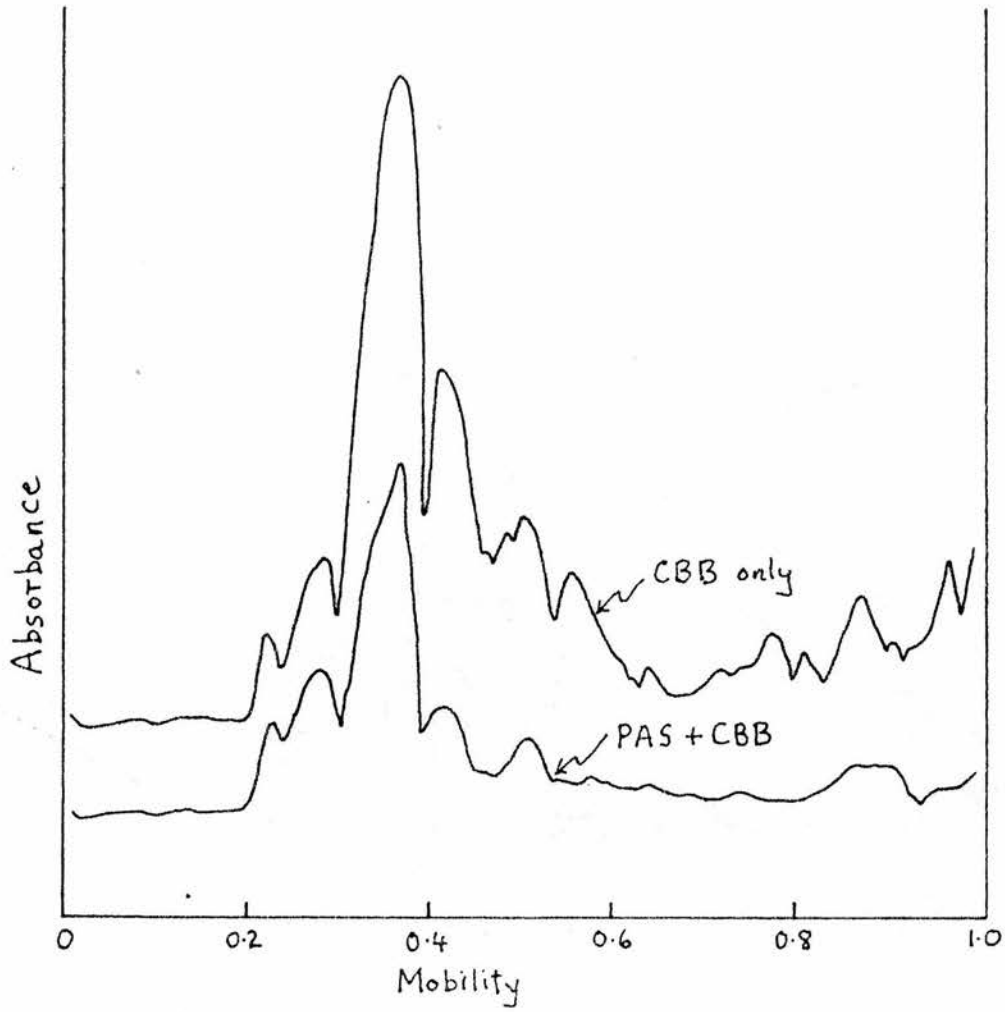


Fig. 4.6 Counter Staining of Dansyl Hydrazine Stained Gels of Chromaffin Granule Lysate with CBB

Following staining with dansyl hydrazine (see Fig. 4.5a) a gel containing reduced chromaffin granule lysate proteins was stained with coomassie brilliant blue. Also shown is the CBB staining profile of an equivalent gel stained immediately after electrophoresis with coomassie brilliant blue only.

stain have been included in the analysis of the glycoproteins of the chromaffin granule membrane but only in a confirmatory basis. It was not considered justifiable to identify proteins as glycoproteins solely on the basis of dansyl hydrazine staining.

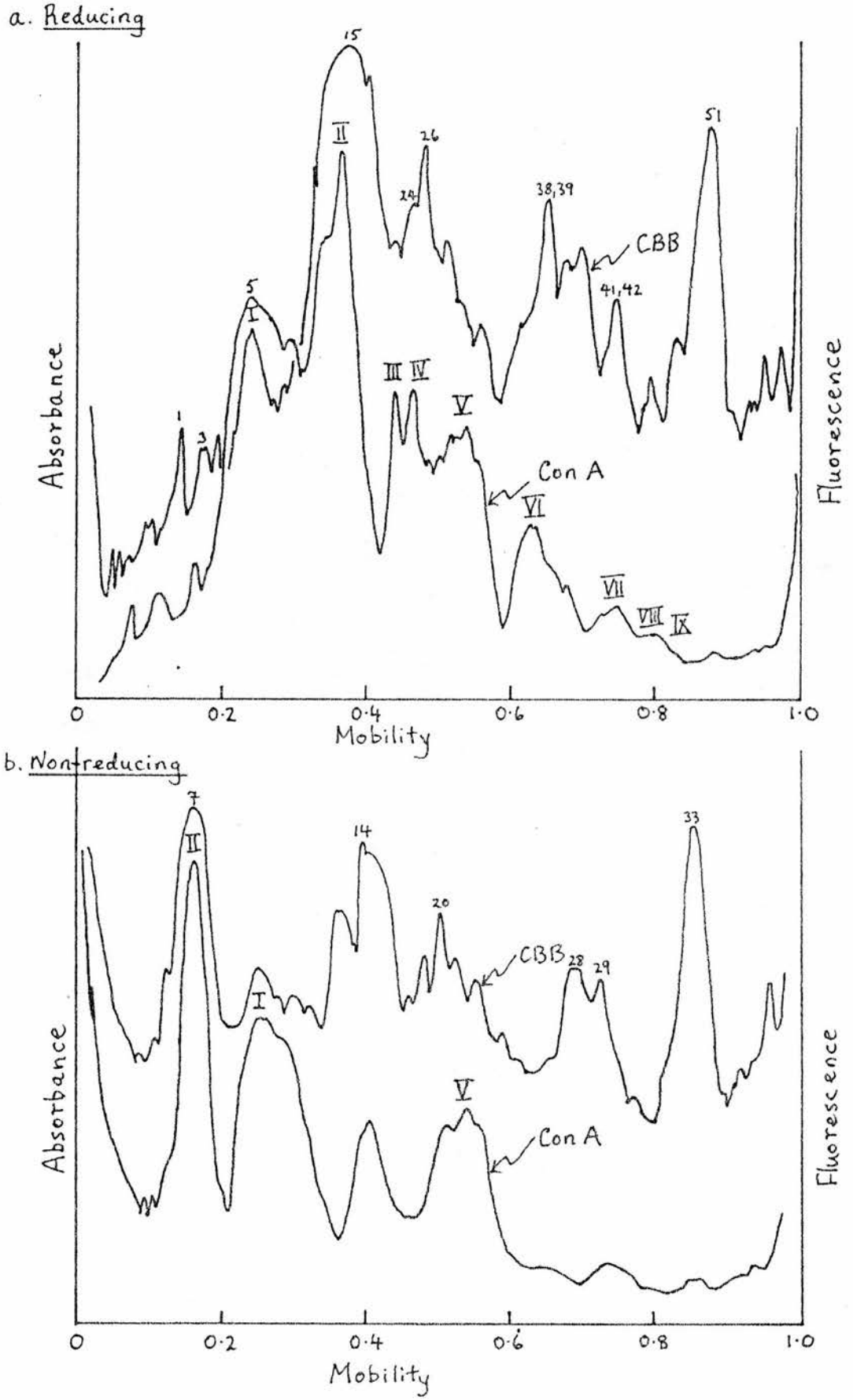
4.3.3 Lectin Staining

The Concanavilin A (Con A) binding sites of reduced granule membranes are indicated in Fig. 4.7a. DβH is the major Con A binding glycoprotein in the gel. This is shown by the co-migration of the major Con A peak with the CBB bands (13, 14 and 15) recognized as containing the DβH subunits and is confirmed by the alteration of its mobility when gels of granule membranes subjected to electrophoresis in the absence of prior reduction are stained with FITC - Con A (Fig. 4.7b). Chromomembrin B does not bind Con A.

The PAS profile and the Con A binding profile of reduced granule membranes are very similar (see Table 4.1) with the differences that exist between them probably being due to the better resolution of the lectin staining technique. Thus PAS band I, II, IV and V correspond to Con A bands I, II, V and VI respectively whilst PAS III has been resolved into the two Con A binding components III and IV. Con A components VII, VIII and IX which are relatively minor Con A binding glycoproteins are not detected by the PAS staining procedure. This may be due to the lesser sensitivity of the PAS stain or to the absence of terminal sialic acid residues in these glycoproteins. Two of these components (Con A VII and VIII) appear to correspond with the DH stained bands VII and VIII respectively (Table 4.1) and the resolution of PAS III into two components (DH III and IV) is also seen in the dansyl hydrazine stained gels.

Fig. 4.7 The Concanavilin A Binding Sites of Chromaffin Granule Membranes

After SDS polyacrylamide gel electrophoresis, gels (10% acrylamide) of chromaffin granule membranes (approx 100 μgms protein) in the reduced (a) and non-reduced (b) state were stained with FITC-Con A. Staining was detected by scanning for fluorescence. The CBB staining profiles of gels of reduced and non-reduced granule membranes is also shown.


a. Reducing
15
II
26
24
38,39
51
CBB
5
I
41,42
III IV
V
3
Con A
VI
VII
VIII IX
Absorbance
Fluorescence
0
0·2
0·4
0·6
0·8
1·0
Mobility
b. Non-reducing
7
II
14
20
33
CBB
28
29
I
V
Con A
Absorbance
Fluorescence
0
0·2
0·4
0·6
0·8
1·0
Mobility

Of the Con A binding glycoproteins, Con A bands I, V, VII, VIII and IX appear to be unaffected by reduction (Fig. 4.7a and 4.7b) whilst Con A III and IV are reduced in intensity and Con A II and VI exhibit altered mobilities. As discussed earlier Con A II(DβH) is the slowest Con A binding component in non-reducing gels of granule membranes. A new component which migrates between Con A II and Con A III in non-reducing gels may be the non-reduced form of Con A VI.

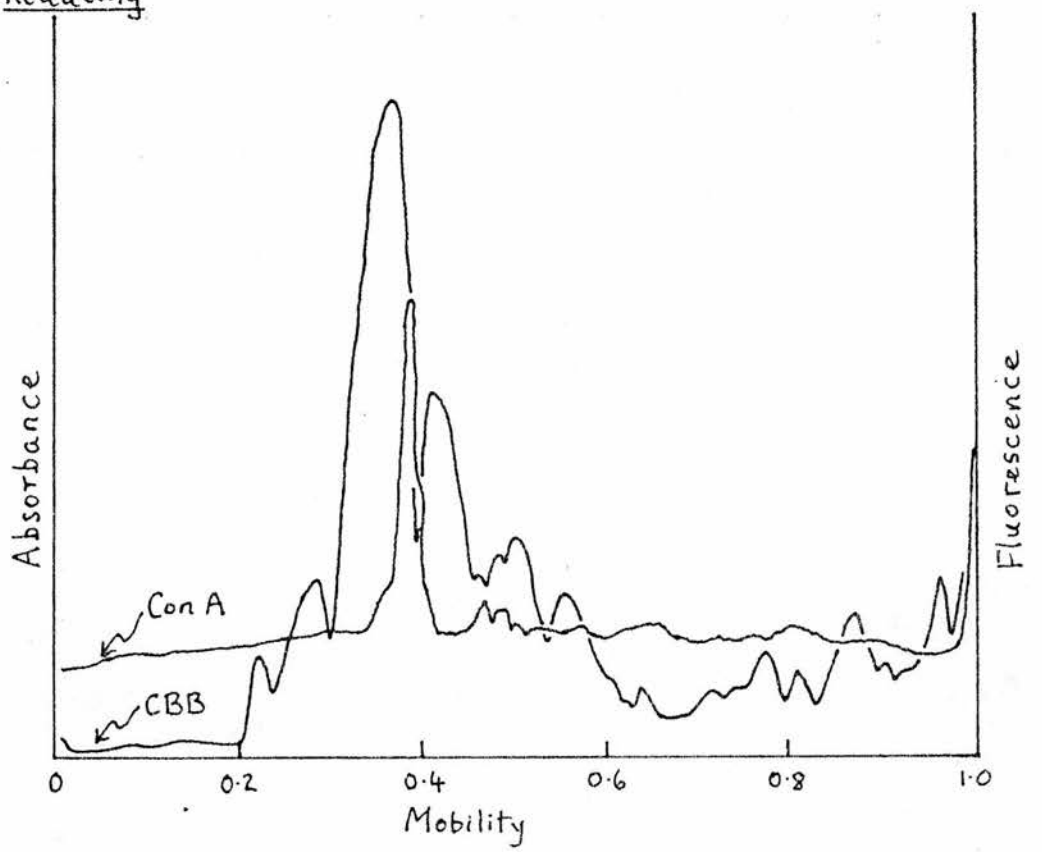
Gels of reduced or non-reduced granule lysate proteins reveal only one component (Fig. 4.8a and 4.8b) viz., dopamine-β-hydroxylase. However, since the majority of the other components are lost from the gel the possibility that other Con A binding sites exist in the lysate cannot be excluded. An interesting feature of the binding of Con A to the reduced form of DβH is the asymmetric nature of the peak (Fig. 4.8a) which appears to be composed of three components present in differing amounts. This asymmetric character is also seen in densitometric scans of CBB stained reducing gels of preparations of DβH prepared by Con A - affinity chromatography from the granule membrane and granule lysate (see section 3.3.4.1) and on this basis reduced DβH was assigned to the reduced CBB stained bands 13, 14 and 15. This confirms the identity of these bands as the subunits of DβH since not only do they bind Con A (and DβH is supposedly the only Con A binding protein in the lysate) but upon electrophoresis under non-reducing conditions these Con A binding bands disappear completely and are replaced by a single band of much slower mobility.

Wheat germ agglutinin (WGA) binding sites are also present in the granule membrane (Fig. 4.9a and 4.9b). Four regions of staining are seen in gels of reduced granule membranes (Fig. 4.9a), three of which are approximately equally well stained whilst the fourth (WGA III)

Fig. 4.8 The Concanavilin A Binding Sites of Chromaffin Granule Lysate

SDS polyacrylamide gel electrophoresis of reduced (a) and non-reduced (b) chromaffin granule lysate (approx. 100 μgm protein in each case) was performed in 10% acrylamide gels. The gels were then stained with either FITC-Con A or coomassie brilliant blue. FITC-Con A binding sites were detected by fluorescence scanning and CBB staining by densitometric scanning.

a. Reducing



b. Non-reducing

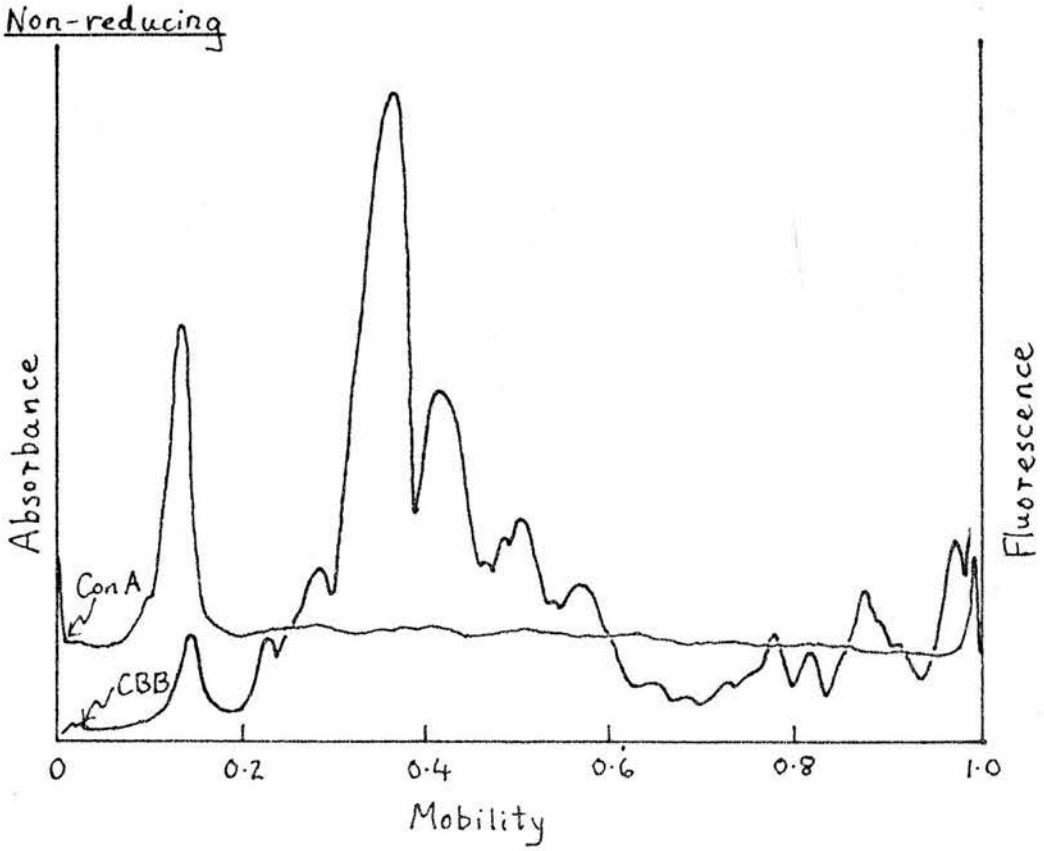
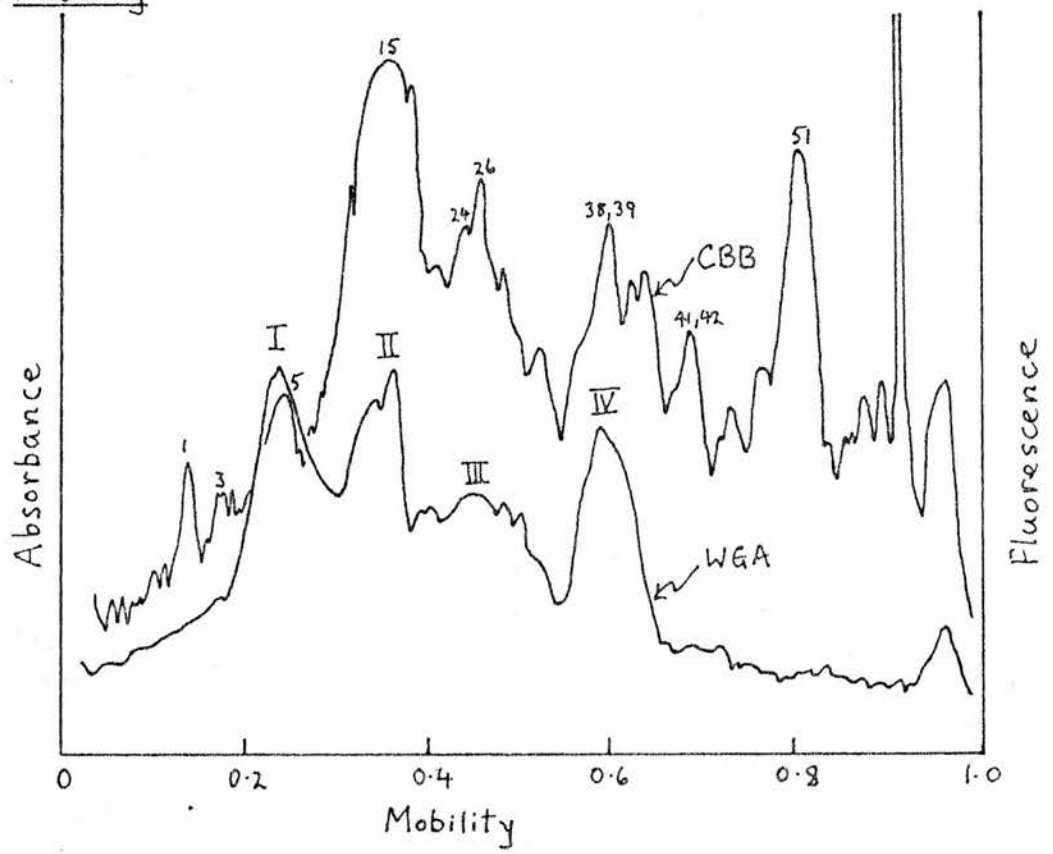


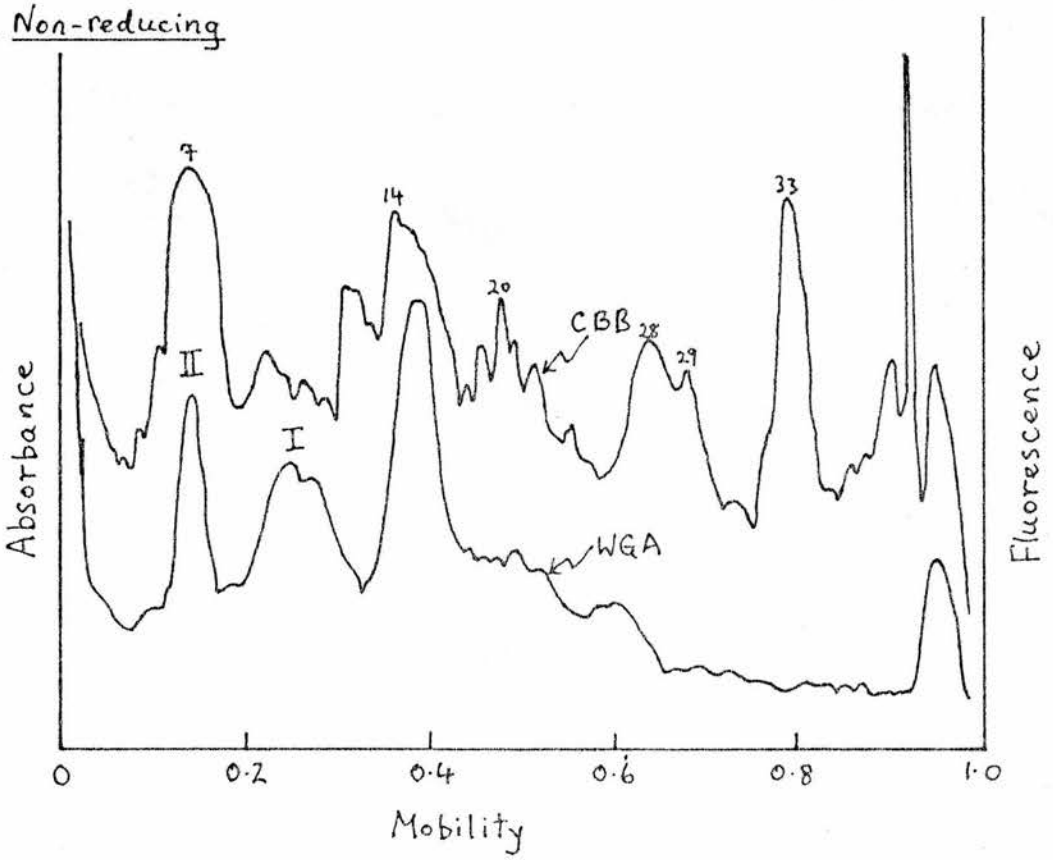
Fig. 4.9 Wheat Germ Agglutinin Binding Sites of Chromaffin Granule Membranes

SDS polyacrylamide gel electrophoresis was performed in 10% (w/v) acrylamide gels. 100 µgm chromaffin granule membrane protein was applied to each lane. Wheat germ agglutinin binding sites were detected by fluorescence scanning of the gels following staining with FITC-WCA. a. Reduced granule membranes; b. Non-reduced granule membranes. The respective CBB staining profiles are also shown.

a. Reducing



b. Non-reducing



is probably poorly resolved and composed of more than one component. WGA I, II and IV probably are identical to the PAS staining glycoproteins I, II (DβH) and V (see Table 4.1) whilst WGA III is composed of PAS bands III and IV.

WGA bands I and III are identified in the same position in non-reducing gels of granule membranes (Fig. 4.9b) as in reducing gels whilst WGA band II, consistent with its identification as DβH, migrates behind WGA I under non-reducing conditions of electrophoresis. The 'new' WGA band migrating immediately behind WGA III is probably the non-reduced form of WGA IV. A trace of WGA IV apparently remains in the position it occuppies under reducing conditions.

In gels of the granule lysate proteins (Fig. 4.10a and 4.10b) DβH is only just stained. No other components in either reducing or non-reducing gels of the soluble proteins can be seen.

In all cases the specificity of lectin binding could be demonstrated by the removal of the lectin from the gel by exposure to the hapten sugar, D-mannose in the case of Concanavilin A and N-acetyl-glucosamine in the case of Wheat germ agglutinin.

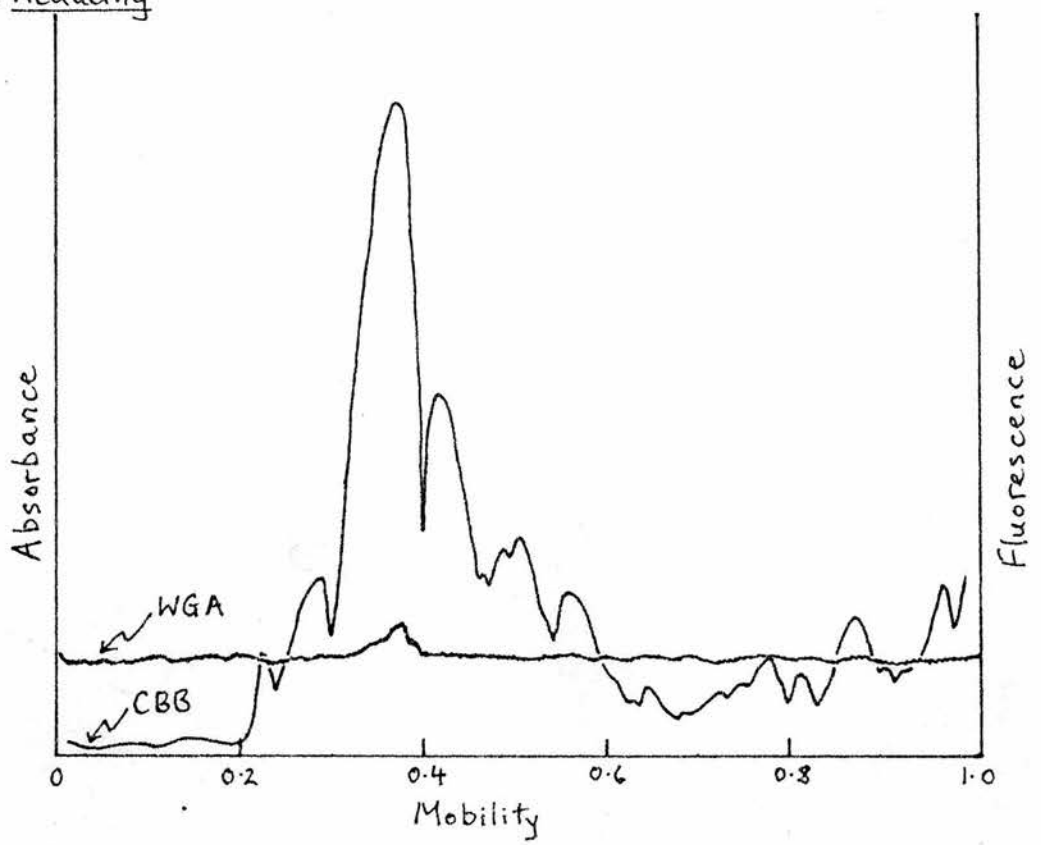
4.3.4 Galactose Oxidase/^{3}H Potassium Borohydride Treatment

Treatment of isolated chromaffin granule membranes with galactose oxidase followed by reduction with ^{3}H borohydride results in labelling of the major glycoproteins of the granule membrane (Fig. 4.11). Galactose Oxidase (GO) bands I, II, IV and V can clearly be identified with PAS bands I, II, IV and V respectively (Table 4.1). GO VI corresponds to the Con A band VII which is not seen in the PAS procedure whilst GO III appears to migrate too close to GO II to be PAS III. When intact granules are subjected to the galactose oxidase/^{3}H borohydride treatment none of the glycoproteins of the granule membrane are

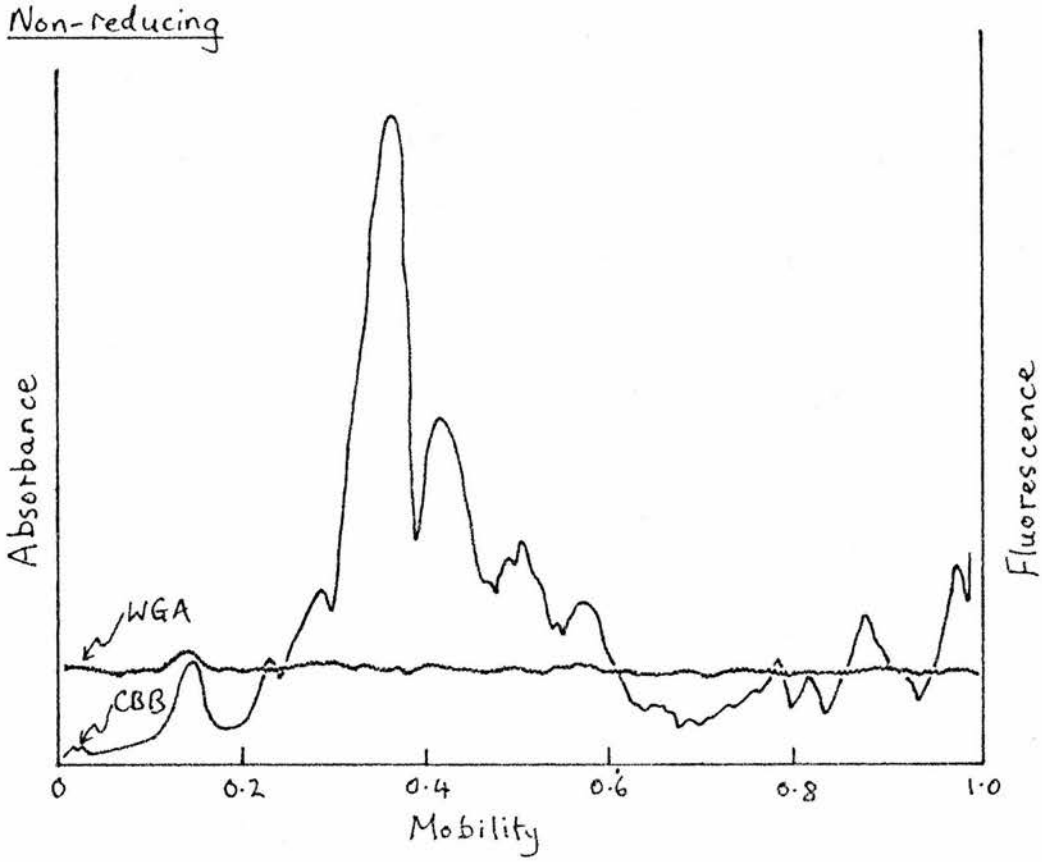
Fig. 4.10 Wheat Germ Agglutinin Binding Sites of Chromaffin Granule Lysate

100 µgm chromaffin granule lysate protein was subjected to SDS polyacrylamide gel electrophoresis in 10% (w/v) acrylamide gels under reducing (a) and non-reducing (b) conditions. After electrophoresis the gels were either treated with FITC-WGA (fluorescence scan) or stained for protein with coomassie brilliant blue (densitometric scan).

a. Reducing



b. Non-reducing



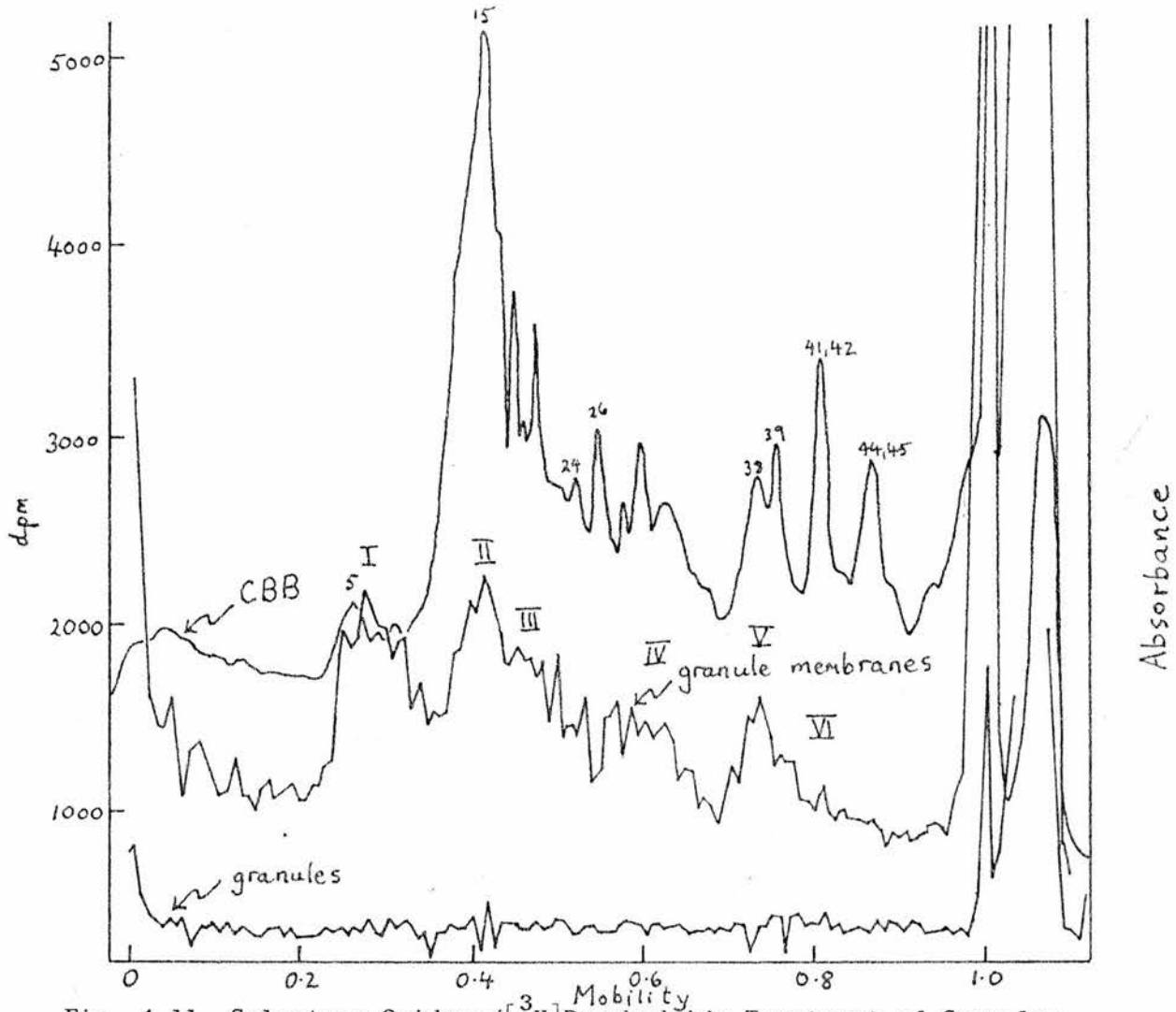


Fig. 4.11 Galactose Oxidase/[^{3}H]Borohydride Treatment of Granules and Granule Membranes

The upper profile ("granule membranes") represents the radioactive distribution in a gel containing granule membranes labelled in the isolated state with [^{3}H]borohydride following galactose oxidase treatment (see section 4.2.5). When granule membranes are prepared from intact granules which have been labelled by the galactose oxidase/[^{3}H]borohydride technique and subjected to electrophoresis the lower radioactive profile ("granules") is obtained. SDS polyacrylamide gel electrophoresis was performed in 8% (w/v) acrylamide gels under reducing conditions. Approximately 100 µgm protein was applied to each lane. Radioactivity was determined by the slicing and counting method (see section 4.2.7).

labelled (Fig. 4.11). The only labelled regions of such gels are the top, tracker dye and lipid region. These three areas are also heavily labelled in gels of granule membranes treated in the purified state and probably represent non-specific labelling.

An interesting feature of this experiment was the slightly altered CBB staining profile of the granule membranes after galactose oxidase and ^{3}H borohydride treatment (Fig. 4.12). The disappearance of bands from the CBB profile of reduced granule membranes is probably due to the action of proteases present in the galactose oxidase preparation. Other workers have noted the presence of proteases in other preparations of this enzyme. Proteolysis was not extensive with CBB stained bands 1, 2, 51 being lost or reduced in intensity when granules were incubated with galactose oxidase and bands 3, 24 affected when isolated membranes were exposed to galactose oxidase. In chapter 5 this finding has been extended in order to localise peptides to the outer surface of the chromaffin granule on the basis of their susceptibility to pronase when granules are incubated with this protease. The appearance of extra bands could be due to degradation products or the presence of adsorbed galactose oxidase in the membrane preparations.

Thus we may conclude that glycoproteins GO I - VI have their carbohydrate moieties exposed on the inner surface of the granule membrane only. It seems unlikely that the presence of a protease in the galactose oxidase preparation has affected the results of this experiment since whilst GO I - VI were clearly available to the galactose oxidase and, therefore, the protease in the isolated granule membrane they were not degraded and therefore must be resistant to proteolysis.

4.3.5 Periodate/^{3}H Potassium Borohydride

Isolated chromaffin granule membranes labelled with ^{3}H after periodate oxidation and ^{3}H borohydride reduction exhibited very poor

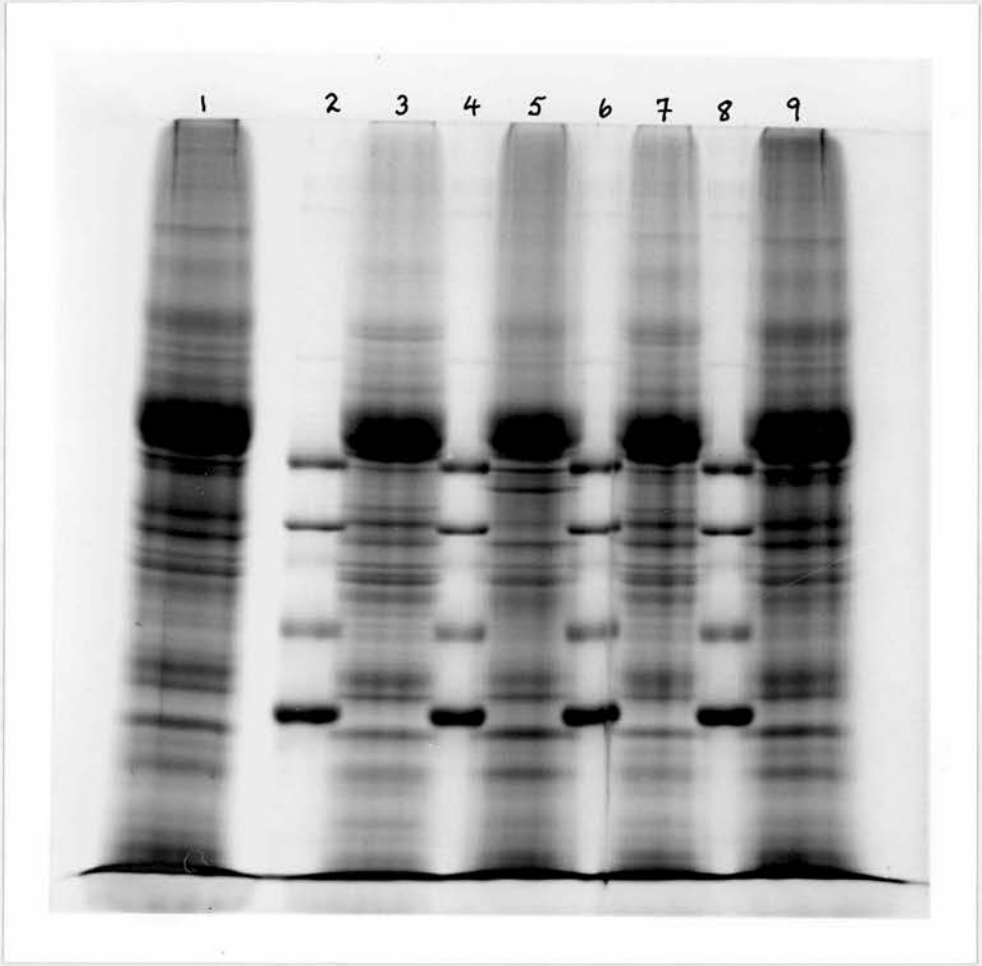


Fig. 4.12 Suspected Proteolytic Activity of the Galactose Oxidase Preparation

Lane 1, untreated granule membranes; lane 3, granule membranes prepared from granules treated with galactose oxidase and [^{3}H]borohydride (see section 4.2.5); lane 5, granule membranes directly treated with galactose oxidase and [^{3}H]borohydride; lane 7, granule membranes prepared from granules oxidized with periodate and reduced with [^{3}H]-borohydride; lane 9, granule membranes directly oxidized with periodate and reduced with [^{3}H]borohydride; lanes 2,4,6 and 8 each contain (in decreasing molecular weight) phosphorylase a, bovine serum albumen, catalase, H-chain γ globulin, ovalbumen, glyceraldehyde phosphate dehydrogenase and L-chain γ globulin. SDS polyacrylamide gel electrophoresis was performed under reducing conditions in an 8% (w/v) acrylamide gel. Approximately 100 µgm protein was applied to each lane. The gel was stained for protein with coomassie brilliant blue.

labelling only one glycoprotein (DβH i.e. PAS II) being detected by this technique (Fig. 4.13). Because of its dependence upon periodate oxidation this method should detect sialoglycoproteins and we have already established that there are at least 5 and possibly 9 sialic acid containing glycoproteins in the chromaffin granule membrane. The failure of this method to detect more than one sialoglycoprotein is probably due to the very mild conditions employed for oxidation (1 mM periodate for 10 min. at $0^{o}C$ compared to 20 mM periodate for 1 hr. at RT for oxidizing gels for the PAS stain) and possibly the fact that oxidation is easier in the denatured state in gels. However, these conditions were those recommended to be mild enough for periodate oxidation to take place only on the outside of enclosed cells or organelles and identical treatment of the membranes with periodate/^{3}H borohydride is required for comparison of their labelling with that of membranes prepared from granules subjected to this labelling technique.

Membranes prepared from periodate oxidized, ^{3}H borohydride reduced intact chromaffin granules failed to label any of the glycoproteins including DβH. Heavy labelling did, however occur at the top of the gel, dye front and in the lipid region of the gel. This is probably non-specific labelling - see Fig. 6., Huber et al. (1979). Thus DβH (or at least its carbohydrate moiety) is not exposed on the outer surface of the granule. Because of the failure of the remaining glycoproteins to be labelled in isolated membranes when they should be readily accessible to periodate oxidation no indication can be gained from this particular experiment regarding their topographical arrangement in the membrane.

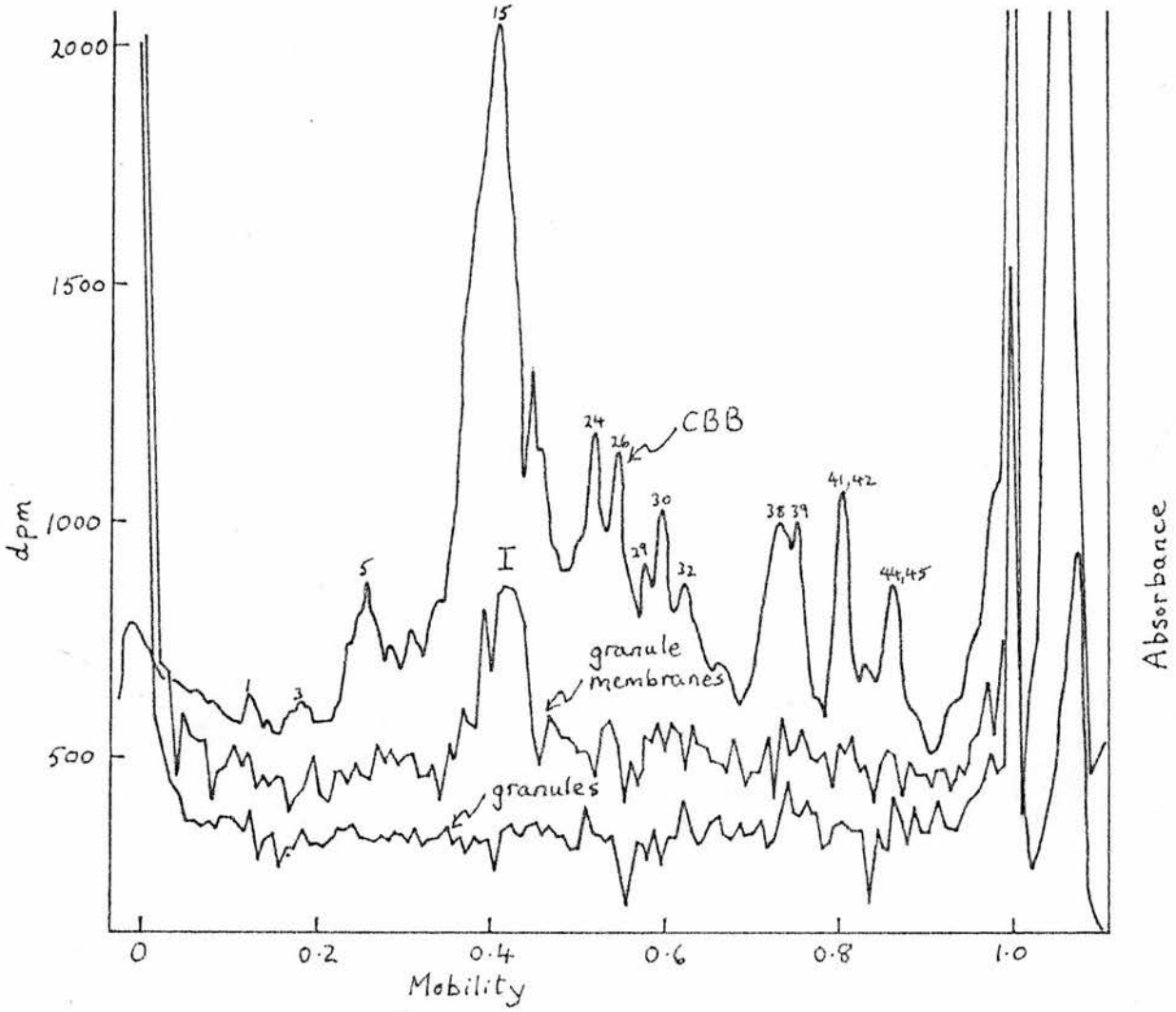


Fig. 4.13 Periodate/[^{3}H]Borohydride Treatment of Granules and Granule Membranes

Intact granules or isolated granule membranes were labelled with [^{3}H]borohydride following periodate oxidation (see section 4.2.6). Membranes (100 µgms per lane) prepared from labelled granules or by washing labelled membranes were subjected to SDS polyacrylamide gel electrophoresis in 8% acrylamide gels under reducing conditions. After staining with CBB radioactivity was determined in gel slices by liquid scintillation spectrometry following solubilization of the gel pieces (see section 4.2.7).

4.4 Discussion

4.4.1 Detection of Glycoproteins

The periodic acid - Schiff, dansyl hydrazine, FITC-Con A and FITC-WGA stains have been used to detect glycoproteins in gels of chromaffin granule membranes and lysate separated by SDS polyacrylamide gel electrophoresis. Glycoproteins of the granule membrane have also been labelled with [^{3}H]-borohydride following oxidation with galactose oxidase or sodium periodate and then subjected to electrophoresis. The specificity of these methods for identifying glycoproteins could be demonstrated in all cases except for the dansyl hydrazine stain. Thus gels not oxidized with sodium periodate failed to stain with Schiff's reagent whilst staining of glycoproteins with FITC-lectins could be inhibited by the hapten sugar of the lectin. Granule membranes prepared from granules treated with galactose oxidase or sodium periodate and then reduced with [^{3}H]-borohydride failed to show any incorporation of tritium into glycoprotein indicating that non-specific binding of [^{3}H]borohydride to glycoprotein did not occur (borohydride is membrane permeant). However, periodate oxidized and non-oxidized gels containing granule membranes or lysate stained equally well with dansyl hydrazine. Although in the main results obtained with dansyl hydrazine were similar to those achieved with other carbohydrate stains components which stained only with dansyl hydrazine were not considered as glycoproteins.

A disadvantage of gel staining techniques to identify glycoproteins is that certain proteins may fail to be fixed in the gel. This is seen in the case of the soluble proteins of the granule where most of the chromogranins, including chromogranin A are eluted from the gel before they can be stained for carbohydrate. The soluble form of DβH is retained within the gel. No coomassie brilliant blue stainable components of chromaffin granule membranes are lost during carbohydrate staining

although it is impossible to determine whether glycoproteins or other components which do not stain with coomassie brilliant blue have been eluted from the gel. Thus all the glycoproteins present in the granule membrane and lysate may not have been identified despite the use of a variety of glycoprotein stains.

Co-migration of two or more glycoproteins would also lead to an underestimate of the number of glycoproteins present. For instance, it appears that GPI (see below)of the granule membrane may be two glycoproteins (GPs Ia and Ib) which co-migrate during electrophoresis in 8% and 10% acrylamide gels but are separated by electrophoresis in gradient gels whilst GP III and GP VII of the membrane run together in non-reducing gels but in reduced gels GP VII migrates much faster than GP III. The occurrence of more than one glycoprotein in each carbohydrate band may explain why so many of these bands stain for different terminal sugars. However, this is not the only reason since DβH clearly shows multiple binding sites for a variety of carbohydrate stains of different specificity. It may be that different populations of a glycoprotein have different carbohydrate chains present or a particular glycoprotein may contain several types of carbohydrate chain within the same molecule. Alternatively carbohydrate chains may be present in different stages of completion or it may be that certain of these stains, particularly the FITC-lectins, can bind internal sugar residues as well as terminal ones.

It is well known that glycoproteins migrate anomalously during SDS polyacrylamide gel electrophoresis. The presence of carbohydrate inhibits SDS binding so that glycoproteins do not migrate at a rate which is a function of the logarithm to the base ten of their molecular weight.

Nevertheless apparent molecular weights based upon their mobility in 8% acrylamide gels under reducing conditions of SDS polyacrylamide gel electrophoresis have been calculated. The extent to which the apparent molecular weight differs from the true molecular weight will depend upon the amount of carbohydrate present, the less carbohydrate the more accurate the molecular weight calculated from mobility.

Sugar residues in glycoproteins also inhibit staining with coomassie brilliant blue especially if the glycoproteins contain a large number of sialic acid residues (Fairbanks et al., 1971; Holden et al., 1971). In Table 4.1 are indicated CBB stained bands of granule membranes which co-migrate with carbohydrate bands of the membrane. It is likely, however, that in the majority of cases the CBB stained bands and carbohydrate bands do not represent the same component.

4.4.2 Glycoproteins of the Chromaffin Granule Membrane

Chromaffin granule membranes contain at least ten glycoproteins ranging in subunit molecular weight 103,000 daltons to 23,000 daltons (see Table 4.1) six of which are major carbohydrate staining components and four minor ones. The major CBB staining component of granule membranes, dopamine-β-hydroxylase (see chapter 3) stains for carbohydrate but chromomembrin B does not appear to be a glycoprotein although it does appear to interact non-specifically with dansyl hydrazine. The presence of several components binding FITC-Con A and FITC-WGA is in agreement with the observations of Eagles et al. (1975) and Meyer and Burger (1976) on the interaction of these lectins with granule membranes.

The slowest moving glycoprotein in reducing gels of granule membranes, GP I, has an apparent subunit molecular weight of approximately 103,000 daltons. Its mobility is unaltered by the absence of dithiothreitol and it is, therefore, not composed of disulphide bonded subunits.

GP I is a major carbohydrate staining component and stains with the PAS reagent, dansyl hydrazine, FITC - Con A and WGA and is labelled with [^{3}H]-borohydride following galactose oxidase treatment indicating that this glycoprotein may contain sialic acid, D-glucose and/or D-mannose, N-acetylglucosamine and galactose and/or N-acetylgalactosamine residues. Only minor sharply resolved CBB staining components are found in the same region of the gel as GP I which characteristically stains as a very broad band. It therefore seems unlikely that GP I stains with coomassie brilliant blue. PAS stained gels containing granule membranes prepared from pronase digested granules (see chapter 5) show that the leading edge of GP I has been degraded. Thus GP I may contain at least two glycoproteins.

GP II has been identified as the membrane bound form of dopamine-β-hydroxylase. In reducing gels of granule membranes DβH can be stained with the PAS reagent, dansyl hydrazine, FITC-Con A and WGA or labelled with [^{3}H]-borohydride following oxidation with galactose oxidase or sodium periodate prior to electrophoresis whilst in non-reducing gels of the membranes DβH has been located with dansyl hydrazine, FITC-Con A and FITC-WGA. The soluble form of DβH can also be detected with the dansyl hydrazine, FITC-Con A and FITC-WGA stains in both reducing and non-reducing gels of the granule lysate.

Other workers have concluded that DβH is a glycoprotein. This finding is based upon sugar analysis of samples of DβH purified from a number of sources (Wallace et al., 1973; Aunis et al., 1974; Ljones et al., 1976; Geissler et al., 1977) and the ability of DβH to bind Concanavilin A. (Rush et al., 1974; Aunis et al., 1975a, 1975b; Ljones et al., 1976). Purified DβH can also be stained by the PAS procedure (Wallace et al., 1973).

Aunis et al. (1975a) found that DβH could not be precipitated by lectins purified from Ricinus sanguinis and Dilochos biflorus which bind D-galactose or N-acetylgalactosamine and N-acetylgalactosamine respectively while Huber et al. (1979) failed to observe any interaction between DβH and Ricinus communis agglutinin (RCA), a lectin specific for N-acetyl-D-galactosamine. This would seem to indicate the absence of D-galactose and N-acetyl-D-galactosamine in DβH. However, Huber et al. (1979) and myself find that DβH can be tritiated with [^{3}H]-borohydride following oxidation with galactose oxidase while Cahill and Morris (1979) showed that both soluble and membrane bound forms of DβH could be labelled with RCA-60 or 120 coupled to fluorescein isothiocyanate. Possible explanations for these differences are that Ricinus sanguinis and Dilochos biflorus lectins interact with DβH but do not cause precipitation and that the presence of sodium dodecyl sulphate interferes with the binding of DβH to RCA - sepharose. In addition, FITC-lectin staining is more sensitive than lectin affinity chromatography for identifying lectin binding components.

In Table 4.2 various reported sugar compositions of DβH are compared with that predicted from the specificity of the various carbohydrate stains and labelling procedures which react with DβH. Although there is considerable disagreement over the carbohydrate content most workers do report sugars which could account for the observed interactions of DβH with FITC - Con A, FITC - WGA, dansyl hydrazine, periodic acid - Schiff reagent, galactose oxidase/[^{3}H]-borohydride and sodium periodate/[^{3}H]-borohydride. Ljones et al. (1976) did not report any glucose or mannose although they used concanavilin A - sepharose affinity chromatography as a part of their purification procedure. Sialic acid was not one of the main sugars in Geissler et al's. (1977) preparation of soluble DβH which is reportedly similar to soluble plus membrane DβH in terms of sugar composition.

	Ljones et al. (1976)	Aunis et al (1974)		Wallace et al (1973)	Geissler et al (1977)
	Soluble + membrane	Soluble	Memb.		Soluble
Galactose	++	++	0	+	+
Glucose	0	+	+	+	
Glucosamine	+++	+	+	++	+
Sialic Acid	+	+++	trace	+	
Mannose	n.d.	+	+	++	+
N-acetyl-galactosamine	n.d.	+	trace		
Fucose	n.d.	0	0	+	+

Table 4.2 Reported Sugar Compositions of Dopamine-β-Hydroxylase

It is uncertain as to whether the soluble and membrane bound forms of DβH have the same carbohydrate composition. Geissler et al. (1977) intimates they do whereas Aunis et al. (1974) find that membrane bound DβH does not contain galactose but other differences between the two forms of the enzyme are quantitative. I cannot make estimates of quantitative differences in the sugar composition of soluble and particulate DβH from the data presented in this chapter but my results are consistent with a similar sugar composition for the two forms of the enzyme.

DβH (both forms) stains with coomassie brilliant blue indicating a relatively small proportion of carbohydrate present. Estimates of 5% carbohydrate or less (Aunis et al., 1974; Wallace et al., 1973; Ljones et al., 1976) for DβH suggest that this is so.

GP III of reduced chromaffin granule membranes, apparent molecular weight 68,000 daltons, contains WGA binding sites and can be labelled with [^{3}H]-borohydride following galactose oxidase treatment. Its position in oxidising gels is obscured by GP VII so it is not possible to assess whether its mobility is affected by dithiothreitol. GP III does not stain with coomassie brilliant blue.

Unlike the majority of glycoproteins in the chromaffin granule membrane GPs IV and V (apparent m. wt. 58,000 and 56,000 daltons respectively) are usually sharply resolved and appear to be CBB staining components 24 and 26 respectively of reduced granule membranes. In non-reducing gels these sharp bands are lost but there is still stain associated with this region of the gel (especially FITC-WGA) so there may be other glycoproteins in this region. It is not certain whether GPs IV and V can be labelled with [^{3}H]- borohydride following galactose oxidase treatment.

CBB stained bands 28 to 35 co-migrate with GP VI - a broadly staining glycoprotein of apparent molecular weight 50,000 daltons which can be identified with periodic acid - Schiffs reagent, dansyl hydrazine, FITC - Con A, FITC-WGA and [^{3}H]-borohydride/galactose oxidase. Its mobility is unaffected by dithiothreitol.

The last major staining glycoprotein of the membrane, GP VII has a reduced molecular weight of 37,000 daltons and one of 68,000 daltons in non-reducing gels. This glycoprotein stains moderately well with coomassie brilliant blue although CBB stained bands, 37, 38 and 39 which co-migrate with GP VII do not represent this glycoprotein. Under certain conditions bands 37, 38 and 39 can be digested with pronase (see chapter 5) to leave a broadly staining CBB band which can still be stained for carbohydrate by the PAS procedure. GP VII can be detected by all the carbohydrate labelling techniques I have employed except for the [^{3}H]-borohydride/sodium periodate method.

GPs VIII, IX and X are minor staining glycoproteins which with the exception of GP VIII are only labelled with the more sensitive dansyl hydrazine and FITC-Con A stains. GP VIII is also detected by the [^{3}H]-borohydride/galactose oxidase procedure.

4.4.3 Glycoproteins of Chromaffin Granule Membranes Characterized by Other Workers

Eagles et al. (1975), Cahill and Morris (1979) and Huber et al. (1979) have also stained gels of chromaffin granule membranes, separated by SDS polyacrylamide gel electrophoresis, for carbohydrate. Eagles et al. (1975) and Huber et al. (1979) used the PAS stain, the latter authors also employed lectin-sepharose affinity chromatography of SDS solubilized granule membranes to determine which of their glycoproteins bound concanavilin A, wheat germ agglutinin and Ricinus communis

agglutinin while Cahill and Morris (1979) stained gels of granule membranes with dansyl hydrazine, FITC-Con A, FITC-WGA, FITC-RCA-60 and FITC-RCA-120. In Table 4.3 I have indicated which glycoproteins characterized by other workers are likely to be the same as the ones I have described.

In general there is a good agreement between the glycoproteins identified by myself and those of other workers (Cahill and Morris, 1979; Huber et al., 1979; Eagles et al., 1975). There are, however, a number of differences. Whilst Cahill and Morris (1979) find that GPs I, II, III, VI and VII (my notation - see Tables 4.1 and 4.3) of the granule membrane can be stained with FITC-RCA-60 or FITC-RCA-120 Huber et al. (1979) could not detect any RCA binding components using lectin affinity chromatography even though they showed that all the major glycoproteins could be labelled with [^{3}H]-borohydride following galactose oxidase treatment. Both RCA and galactose oxidase require the presence of galactose or N-acetylgalactosamine for interaction. Huber et al. (1979) also found fewer Con A and WGA binding sites than myself and Cahill and Morris (1979). Thus it may be that lectin affinity chromatography is not as sensitive as lectin staining or that the presence of SDS during lectin affinity chromatography interfers with the binding of certain glycoproteins to lectins.

Eages et al. (1975) show two PAS staining components which migrate slower than DβH in reducing gels. These may represent GP Ia and Ib, components of GP I.

4.4.4 Glycoproteins of the Chromaffin Granule Lysate

Staining of gels of the soluble proteins of chromaffin granules separated by SDS polyacrylamide gel electrophoresis reveals only one glycoprotein. From its mobility under non-reducing and reducing conditions

M. Abbs	Huber et al. (1979)	Cahill and Morris (1979)	Eagles et al. (1975)
		185	
		148	
I	II	100	1, 2?
II	I	75	3
II or III?	III	69	
IV		59	
V		55	
		51	
VI	IV	47	4?
VII	V	36	5?
VIII			
IX			
X		25	

Table 4.3 Glycoproteins of the Chromaffin Granule Membrane: Comparison with Other Workers' Results.

of electrophoresis and its ability to bind Con A this glycoprotein appears to be the soluble form of DβH. Dansyl hydrazine and WGA also stain soluble DβH. Cahill and Morris (1979) also find DβH is the only glycoprotein of the granule lysate and that it can be detected by the above carbohydrate stains as well as FITC-RCA-60 and FITC-RCA-120. In addition to DβH, Huber et al. (1979) found another PAS staining component but this was not chromogranin A.

I have been unable to confirm reports that chromogranin A is a glycoprotein (Smith and Winkler, 1967b; Bartlett, see Winkler and Smith, 1975; Geissler et al., 1977) since like several other chromogranins this protein does not appear to be fixed in the gel. Cahill and Morris (1979) also noted this phenomenon whilst Huber et al. (1979) did not report counter-staining their gels with CBB following PAS staining. It has been suggested that chromogranin A is not a glycoprotein (Aunis et al., 1975b).

It is difficult to explain why Cahill and Morris (1979) and myself do not observe the second PAS staining component in Huber et al's (1979) gels of the soluble proteins. Staining with dansyl hydrazine has the same specificity as the PAS procedure. It may be that this glycoprotein was eluted from my gels and those of Cahill and Morris (1979) prior to carbohydrate staining because of differences in the fixation procedure used (Fairbanks et al., 1971; Eckhardt et al., 1976).

4.4.5 Topography of Glycoproteins in Chromaffin Granule Membranes

The major glycoproteins of the chromaffin granule membrane can be labelled with [^{3}H]-borohydride following treatment of the broken membranes with galactose oxidase. When intact granules are used none of the glycoproteins bcomes labelled. Membrane glycoproteins also fail to be labelled when intact granules are reduced with [^{3}H]-borohydride

following oxidation with sodium periodate although only DβH is labelled when broken membranes are used. These observations are consistent with the view that the carbohydrate moieties at least of the membrane glycoproteins face the internal milieu of the granule.

Huber et al. (1979) also suggest that protein bound carbohydrate of the granule membrane is exposed only at the inner surface. They also used the [^{3}H]borohydride/galactose oxidase technique and confirmed the finding of Eagles et al. (1975) that concanavilin A will bind only to the inner surface of the granule membrane.

Meyer and Burger (1976), however, showed that wheat germ agglutinin could agglutinate granules indicating that sugars may be exposed at the cytoplasmic surface of the granule. Cahill and Morris (1979) repeated Meyer and Burger's (1976) experiments and indicated that the granules are very fragile under the conditions used for agglutination so that sugars present at the inner surface of the membrane may have been available for binding to WGA.

Synaptin, a membrane glycoprotein of synaptic vesicles with a molecular weight of 45,000 daltons (Bock, 1978) has been shown to be present in chromaffin granule membranes and would appear to be exposed at the outer surface of the granule (Bock and Helle, 1977). There was no direct evidence, however, that the carbohydrate moiety was also present at the cytoplasmic surface of the membrane.

CHAPTER 5

CONTROLLED PROTEOLYTIC DIGESTION OF INTACT CHROMAFFIN GRANULES

CONTROLLED PROTEOLYTIC DIGESTION OF INTACT CHROMAFFIN GRANULES

5.1 Introduction

Bender et al. (1971) have shown that controlled proteolytic digestion of intact erythrocytes by proteases can be used to investigate the arrangement of the proteins of the erythrocyte membrane. Proteases, by virtue of being proteins, are deemed sufficiently large to be unable to pass through biological membranes. Pronase, a mixture of proteases from Streptomyces griseus having a broad peptide specificity (Nomoto et al., 1960 a, b; Trop and Birk, 1970), was used. Under the conditions used by Bender et al. (1971) lysis, as revealed by haemoglobin release, was not a serious problem. After digestion the effect of proteolysis on various functions of the erythrocyte membrane, such as acetylcholinesterase activity and glucose transport, was measured. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate was performed on ghosts prepared from the digested erythrocytes in an attempt to correlate any changes in the functions of the erythrocyte membrane with those occuring in the electrophoretic pattern of the ghosts.

It is well known that solubilization of proteins with SDS renders them extremely susceptible to proteolysis and, as pointed out by Bender et al. (1971), precautions must be taken when using SDS gel electrophoresis to avoid any proteolysis during sample preparation by contaminating traces of protease.

The stategy used to probe the arrangement of the membrane proteins of the chromaffin granule with pronase was essentially that used by Bender et al. (1971) i.e. digestion of intact granules with pronase, purification of the granules with concomitant removal of pronase, membrane preparation,

enzyme assays and SDS gel electrophoresis of the membranes. As an extra safeguard against proteolysis occurring outwith the incubation period 'high' concentrations of pronase were avoided. Thus the extent of digestion of the granules was controlled by the length of incubation of the granules with a fixed low concentration of pronase rather than by exposing them to higher pronase concentrations.

5.2 Materials and Methods

5.2.1 Materials

Protease, from Streptomyces griseus, was purchased from Sigma (Pronase, Sigma VI, lot 56C 0013) and potassium biphthalate from BDH (Analar grade).

5.2.2 Digestion of Intact Granules

P_2 granules (2 ml, 10 mg/ml protein) in 0.3 M sucrose, 10 mM hepes, pH 7 were digested with pronase at room temperature for various periods of time. Incubations were terminated by the addition of 10 ml ice cold bufferred sucrose followed by centrifugation at 18,000 g-av for 20 min. at $2^{o}C$. The pellets were each resuspended in 2 ml 0.3 M sucrose, 10 mM hepes, pH 7 and granules purified from them by sedimentation through 1.8 M sucrose, 10 mM hepes, pH 7 (see section 2.1.2). Granule membranes were then prepared by the normal procedure and stored frozen in 10 mM hepes, pH 7.

All incubations were timed to end simultaneously. Control granules were incubated at room temperature in the absence of pronase. In experiments where the extent of digestion was controlled by the length of time of exposure to pronase the granules were pre-incubated for varying periods of time at room temperature such that they were all exposed at room temperature for the same length of time.

5.2.3 Digestion of Granule Membranes

Granule membranes, 1 mg/ml protein in 10 mM hepes, pH 7 were digested at room temperature with pronase. After dilution with 10 ml 10 mM hepes, pH 7, the digested membranes were centrifuged at 160,000 g-av for 1 hr at 2°C. The membranes were washed twice more with 10 mM hepes, pH 7 before final resuspension in this buffer. Membranes used for digestion had previously been frozen and thawed.

5.2.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis in 8% - 20% acrylamide gradient gels was performed as previously described (see section 2.2.2) except for a modification to the composition of the sample buffer. This consisted of the replacement of tris in the sample buffer with 50 mM potassium biphthalate giving a pH of 4, as recommended by Bender et al. (1971), to prevent proteolysis during SDS solubilization. In addition a 2 to 1 mixture of the biphthalate sample buffer and water was preheated in a boiling water bath before the addition of 1 part sample and further incubation at 100°C.

Gels were stained for protein with coomassie brilliantblue (see section 2.2.1) and for carbohydrate by the periodic acid - Schiff procedure (see section 4.3.1).

5.2.5 Other Methods

See chapter 2 for the estimation of protein and cholesterol and the assay of dopamine-β-hydroxylase, Mg^{++} ATPase, NADH:(acceptor) oxidoreductase, phosphatidylinositol kinase and cytochrome b-561.

5.3 Results

Because of the removal of protein from the membrane by pronase treatment, protein cannot be used as a measure of the amount of membrane

present. Instead cholesterol, which should be unaffected by pronase digestion, was used to quantitate the digested membranes. Thus all enzyme activities are expressed in terms of cholesterol content.

5.3.1 Digestion of Intact Granules

5.3.1.1 Lysis

The extent of lysis (see Table 5.1) was measured by the yield of membranes, quantified by cholesterol measurement, recovered from digested granules. Only intact granules will sediment through 1.8 M sucrose. When granules are incubated with 0.1 mg/ml pronase 30% of the membrane protein can be removed after 4 hr digestion. Very little lysis was observed. After much longer incubation with pronase (14 and 24 hr) substantial lysis occurred despite the removal of very little more protein from the granule membrane. It may be that the protein digested in this period is essential for granule stability or that digested granules are much less stable at room temperature than native granules.

5.3.1.2 Effect on CBB Staining Profile

The coomassie brilliant blue staining pattern of granule membranes separated by SDS polyacrylamide gel electrophoresis under reducing conditions in 8% - 20% acrylamide gradient gels was unaffected by sample preparation at pH 4 (see Fig. 5.1) or by incubation of granules for up to 24 hr at room temperature prior to granule membrane preparation (Fig. 5.3 lanes 1 and 4). This pattern is described in section 3.3.1.3

Marked changes in the CBB staining profile of the membranes of granules digested for 10 min, 1 hr and 4 hr with 0.1 mg/ml pronase at room temperature were observed (Fig. 5.1 and 5.2). No further changes occurred with longer periods of digestion (Fig. 5.3) with this pronase concentration. These changes, which are catalogued in Table 5.2., con-

Digestion Period	Cholesterol Recovery (mg/ml)	Protein Recovery (mg/ml)	Protein: Cholesterol
Expt A			
0	4.13 (100%)	4.4	1.06 (100%)
10 min	3.9 (96.6%)	4.1	1.05 (98.6%)
1 hr	4.1 (99.4%)	3.9	0.95 (89.2%)
4 hr	3.6 (87.3%)	2.8	0.78 (72.9%)
Expt B			
0	3.5 (100%)	4.4	1.26 (100%)
4 hr	3.5 (100%)	3.7	1.06 (84.6%)
14 hr	2.4 (68.6%)	1.86	0.78 (61.6%)
24 hr	1.8 (51.4%)	1.5	0.83 (66.3%)

Table 5.1 Lysis During Digestion of Chromaffin Granules

Equal amounts of granules were digested with pronase for various periods of time (see text). Membranes were prepared from the digested granules. Lysis is indicated by the decrease in recovery of membrane cholesterol and the extent of digestion by the fall in the protein to cholesterol ratio of the membranes.

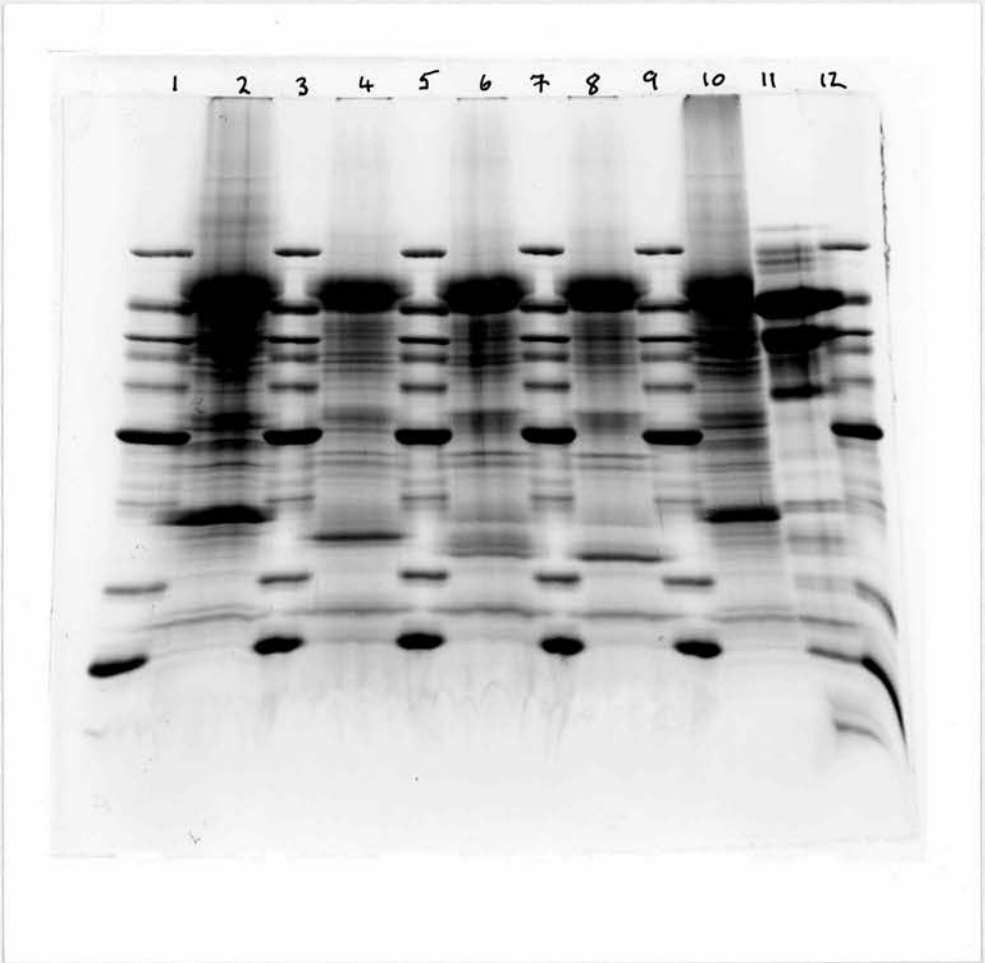
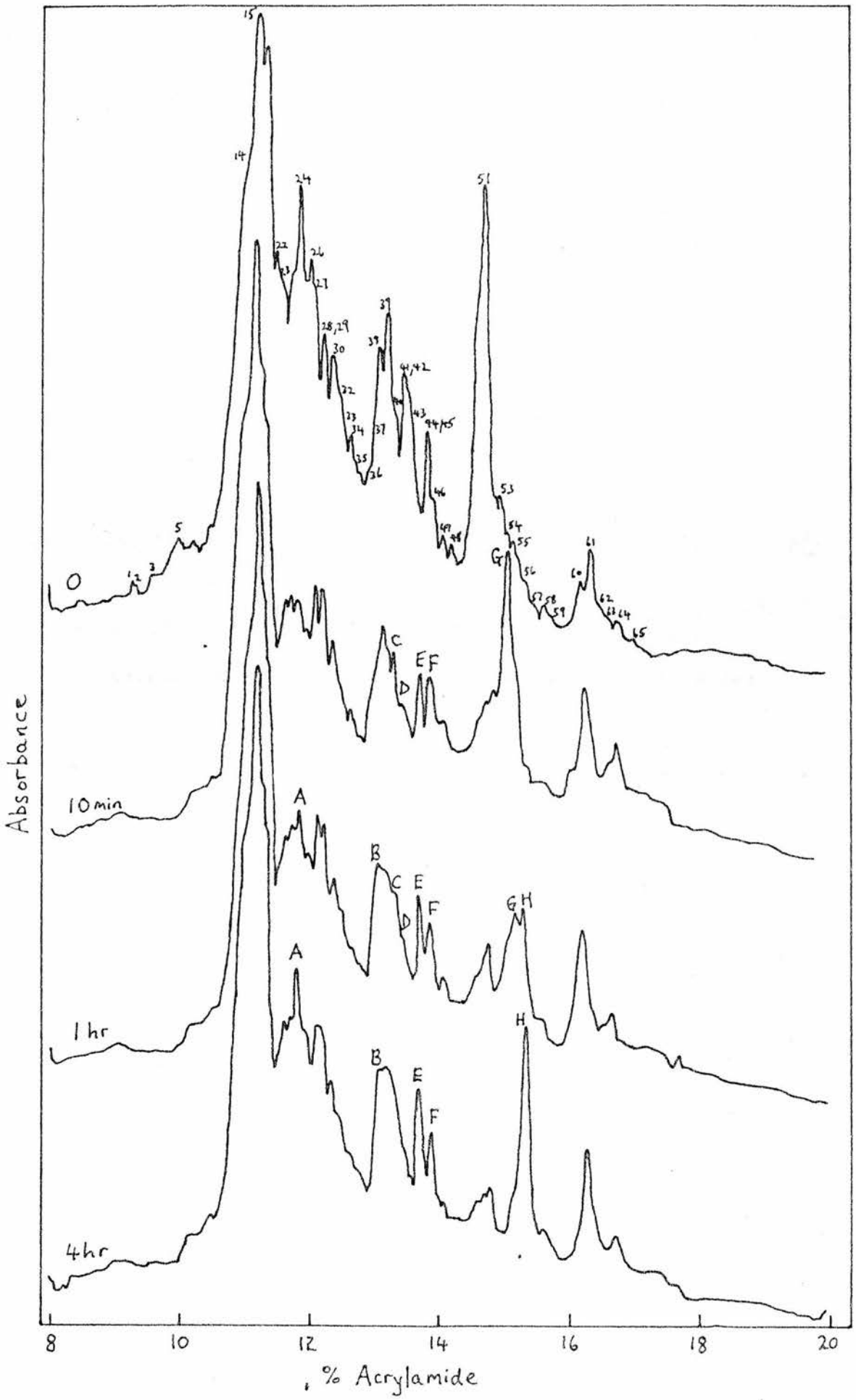


Fig. 5.1 "Digestion of Intact Granules with 0.1 mg/ml Pronase Over 4 h"

P_2 granules (10 mg/ml protein) were digested with 0.1 mg/ml pronase at room temperature for 0 min, 10 min, 1 h and 4 h. Membranes prepared from the digested granules were subjected to SDS polyacrylamide gel electrophoresis in an 8% - 20% (w/v) acrylamide gradient gel under reducing conditions. The gel was stained for protein with coomassie brilliant blue. Equal amounts of membrane(100 µgm cholesterol) were applied to each lane. Lane 2, membranes prepared from granules incubated at room temperature for 4 h in the absence of pronase (control); 4, membranes from 10 min digested granules; 6, membranes from 1 h digested granules ; 8, membranes from 4 h digested granules; 10, granule membranes; 11 granule lysate. Lanes 1,3,5,7,9,12 contain molecular weight markers (phosphorylase a, bovine serum albumen, catalase, H chain γ globulin, ovalbumen, glyceraldehyde phosphate dehydrogenase, L-chain γ globulin, soya bean trypsin inhibitor and cytochrome c).

Fig. 5.2 Pronase Digestion of Intact Granules

Intact granules were digested with pronase and their membranes purified and subjected to SDS polyacrylamide gel electrophoresis as described in Fig. 5.1. Lane 2 (control), 4 (membranes from 10 min digested granules), 6 (membranes from 1 h digested granules) and 8 (membranes from 4 h digested granules) of the gel shown in Fig. 5.1 were densitometrically scanned at 570 nm and contain equivalent amounts of membranes.


Absorbance
0
10 min
1 hr
4 hr
A
B
C
D
E
F
G
H
8
10
12
14
16
18
20
% Acrylamide

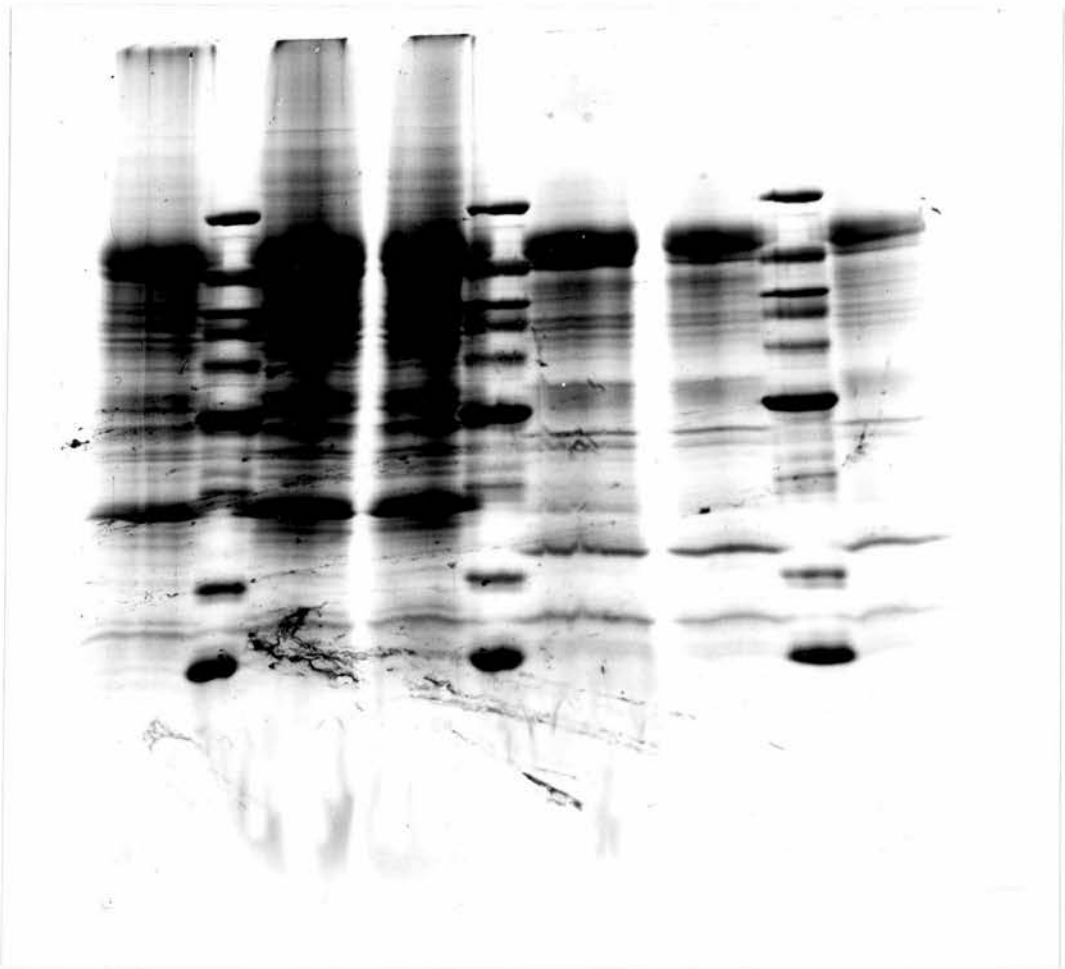


Fig. 5.3 Prolonged Digestion of Intact Granules with 0.1 mg/ml Pronase

Granules were digested with pronase (0.1 mg/ml) for 0 to 24 h at room temperature. Lane 1, granule membranes; lane 3, granule membranes prepared from P_2 granules incubated at $0^{o}C$ for 24 h; lane 4, granule membranes from granules incubated at room temperature for 24 h; lane 6, membranes from granules digested for 4 h; lane 7, membranes from granules digested for 14 h; lane 9, membranes from granules digested for 24 h. Lanes 2, 5 and 8 contain molecular weight markers. Lanes 7 and 9 had less membrane amounts applied (cholesterol content) than the other lanes containing granule membrane preparations.

CBB	M.Wt (10^{-3}D)		Comment
1	165	S	Gone after 10 min.
2	160	S	
3	142	S	Digested but v. faint trace remains after 4 hr.
4	120	S	Gone after 10 min
5	120	S	
6	120	S	
7	110	?	
8	106	?	
9	97	?	
10	97	?	
11	93	?	
12	93	?	
13	77	R	DβH subunits. Resistant
14	72	R	
15	68	R	
16		R	Resistant
17		R	Resistant
18		S	Sensitive - digested in 10 min
19		S	
20	67	S	Gone after 10 min
21	66	R	Resistant
22	65	PR	Steady reduction in intensity
23	63	S	Sensitive, 10 min.
24	59	PR	Digested but new product appears?
25	57	R	Resistant
26	55	S	Digested after 10 min
27	53	R	Resistant
28	51	PR	ATPase subunit, reduced in intensity
29	49	PR	ATPase subunit, reduced in intensity
30	47	PR	Partially digested
31	47	PR	Partially digested
32	46	PR	Partially digested
33	44	?	
34	42	S	Digested - only faint trace remains after 1 hr
35	41	S	Digested after 10 min.
36		?	
37	39	PR	Probably digested, new Band appears after 1 hr — Diffuse staining remains after removal of sharper bands (due to GP VII?)
38	37	PR	
39	35	PR	Probably digested
40	34	?	Obscured by new product itself degraded?
41	33	S	Digested in 1 hr
42	31	S	Digested in 10 min.
43	30	?	Obscured by new band? Increased intensity
44	28	?	Obscured by new band
45	28	S	Digested by 10 min.
46	26	?	
47	26	S	Sensitive - only a trace remains after 4 hr.
48	24	S	Digested after 1 hr.
49	24	S	Chromembrin B. Degraded within 10 min.
50	22	S	tho' trace remains after 4 hr. Appears to
51	21	S	behave as a unit.
52	21	?	
53	20	S	Digested 10 min.
54	20	S	Digested after 4 hr.
55	19	?	Obscured by new band.

CBB	M.Wt (10^{-3}D)		Comment
56	18	S	Digested, 10 min.
57	18	S	Digested, 10 min.
58	17	?	Obscured by broader faint band.
59	16	S	Sensitive 10 min.
60	15	S	Sensitive 10 min.
61	14	R	Resistant
62	14	?	Bands too faint to notice any reduction
63	13	?	in intensity.
64	12	R	Resistant
65	11	S	Sensitive, 10 min.
66	11	?	

Table 5.2 Effect of Pronase Digestion of Intact Granules on the Polypeptide Profile of the Granule Membrane.

For experimental details see Fig. 5.1 and text. CBB band numbers refer to those of reduced granule membranes and the m. wts. (10^{-3} daltons) are those calculated from acrylamide gradient gels.

Key: S - sensitive
R - resistant
PR - partially reduced in staining intensity
? - sensitivity uncertain.

sist of the disappearance of certain staining bands, the appearance of new staining bands and an increase or decrease in the relative staining intensity of some of the bands in the profile.

Bands which are missing from the CBB staining profile of granule membranes from pronase digested granules include those representing chromomembrin B, which is the second most heavily stained region of gels of granule membranes stained with CBB. Chromomembrin B appears to be firstly degraded to degradation band G (molecular weight 19,500 daltons) which in turn is digested to band H (molecular weight 17,500 daltons). Bands G and H were recognized as being derived from chromomembrin B because of the similarity of staining intensity. Other new bands (A,B,C,D,E,F,I, and J - see Fig. 5.2) cannot be assigned to a specific CBB staining component of the granule membrane from which they were derived. New bands, in certain cases obscure original bands of the granule membrane so that the pronase susceptibility of the latter bands cannot be determined.

CBB stained bands 28, 29 and 44 have been identified as subunits of the Mg^{++} ATPase whilst bands 13, 14 and 15 represent dopamine-β- hydroxylase (see section 3.3.4.). All three subunits of the Mg^{++} ATPase show a reduction in staining intensity following pronase digestion of granules although these bands still remain after digestion is complete. Dopamine-β-hydroxylase is unaffected by pronase when granules are digested. Even after extensive digestion, resulting in lysis of 50% of the granules, CBB stained bands 13, 14 and 15 show no reduction in staining intensity.

However, if granules are digested with pronase at a concentration of 0.5 mg/ml instead of 0.1 mg/ml, for half an hour at room temperature DβH is partially degraded to a component (band K) of molecular weight

59,000 daltons (Fig. 5.4). Other differences in the suceptibility of CBB staining components of the granule membrane to pronase digestion in granules at this pronase concentration were observed, e.g. the complete degradation of CBB staining components in the band 37-39 region. It seems likely that these differences are observed because of pronase digestion outside the incubation period since although the gel patterns indicate a greater degree of digestion than with 0.1 mg/ml pronase for 24 hr at room temperature no lysis was observed with digestion at 0.5 mg/ml pronase for ½ hr at room temperature. In the former case (0.1 mg/ml pronase for 24 hr at room temperature) 50% lysis had occurred. Thus emphasis was placed on the use of low concentrations of pronase and increasing incubation periods to control the extent of digestion rather than by increasing the pronase concentration.

5.3.1.3 Effect on Carbohydrate Staining Profile

Close examination of gels of granule membranes prepared from granules digested with 0.1 mg/ml pronase (Fig. 5.5) revealed that the leading edge of PAS band I was missing. Thus PAS band I represents more than one glycoprotein one of which is exposed on the outside of the granule. The remainder of the PAS staining profile was the same as that previously described for granule membranes prepared from untreated granules (see section 4.3.1.). No new PAS staining components were observed. It should be noted, however, that relatively small changes in staining intensity would be difficult to see because of the diffuse nature of the PAS stain.

5.3.1.4 Effect on Enzyme Activities

Fig. 5.6 shows the effect of the digestion of granules with 0.1 mg/ml pronase over a period of 24 hr on the activities of four membrane bound enzymes, dopamine-β-hydroxylase, Mg^{++} ATPase, phosphatidylinositol

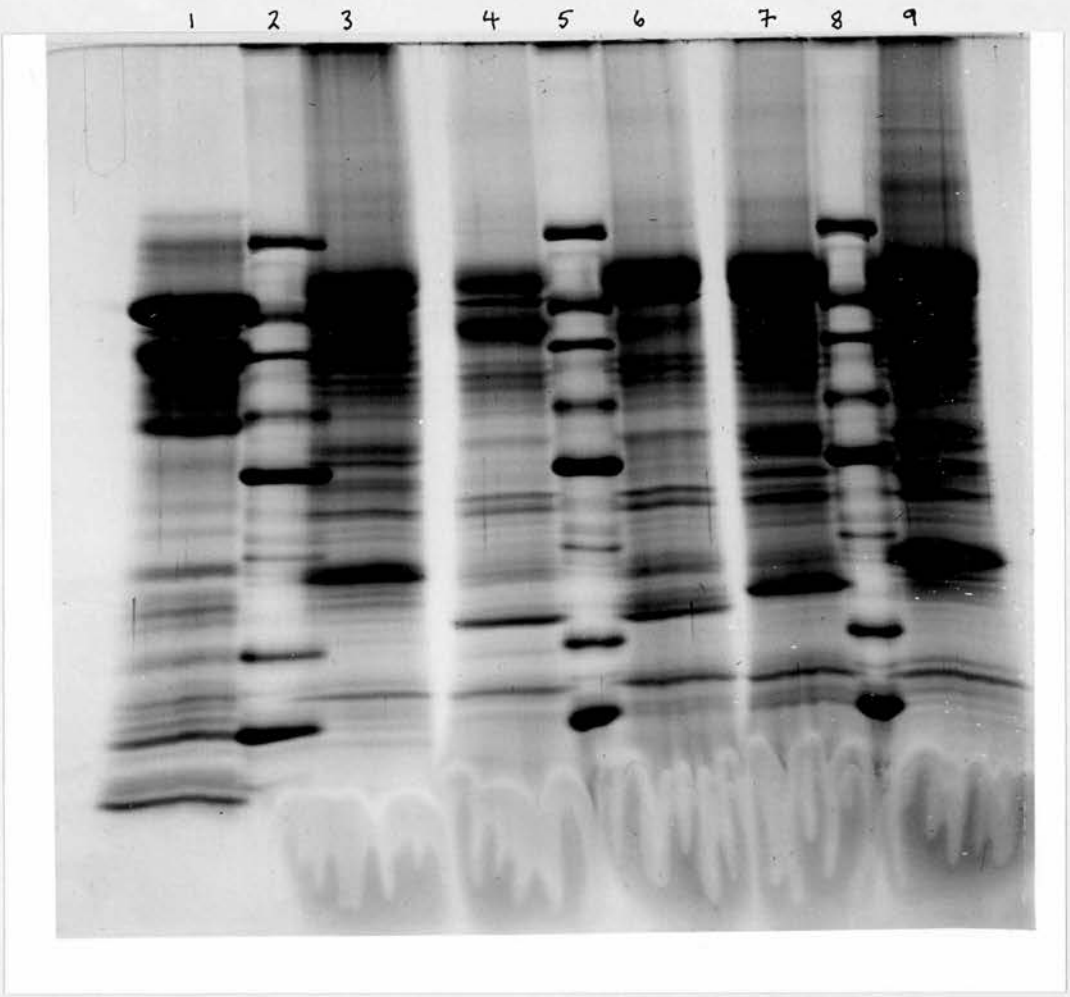


Fig. 5.4 Digestion of Granules with Increasing Concentrations of Pronase

Granules were digested at room temperature for ½ h with increasing concentrations of pronase. Lane 1, granule lysate (0.1 mg protein); lane 3, granule membranes (0.1 mg protein, 0.08 mg cholesterol); lane 4, membranes from granules digested with 0.5 mg/ml pronase; lane 6, membranes from granules digested with 0.2 mg/ml pronase; lane 7, membranes from granules digested with 0.02 mg/ml pronase; lane 9, membranes from granules incubated at room temperature for ½ h in the absence of pronase. Lanes 4,6,7 and 9 each contained the same amount of membrane (0.1 mg cholesterol). Lanes 2, 5 and 8 contain molecular weight marker proteins.

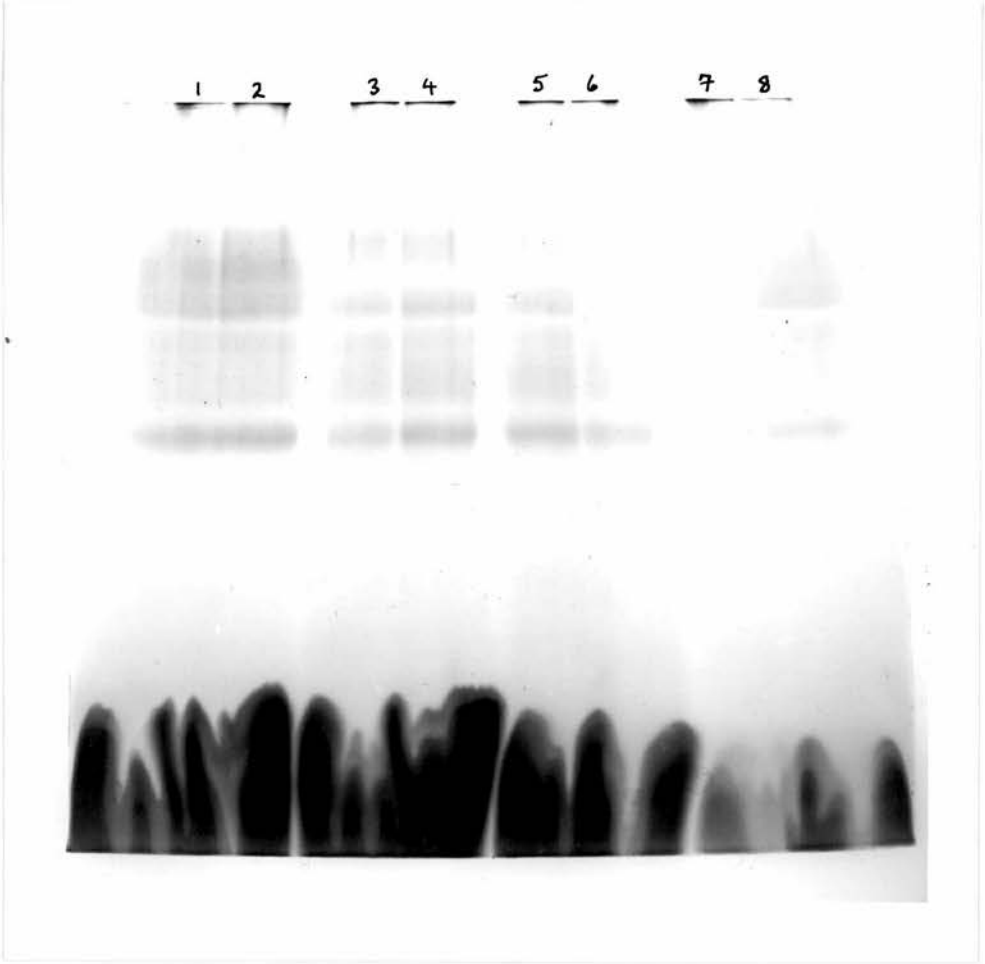


Fig. 5.5 Effect of Pronase Digestion of Granules on the PAS profile of the Granule Membrane

Granules were digested with pronase (0.1 mg/ml) at room temperature for 0 to 24 h. Membranes were prepared and subjected to SDS polyacrylamide gel electrophoresis in 8% - 20% (w/v) acrylamide gels under reducing conditons. The gel was then stained for carbohydrate by the periodic acid - Schiff procedure (see section 4.3.1). Lane 1, granule membranes from granules incubated at $0^{o}C$ for 24 h; lane 2, membranes of granules incubated at room temperature for 24 h; lane 3, membranes of granules digested for 10 min; lane 4, membranes of granules digested for 1 h; lane 5, membranes of granules digested for 4 h; lane 6, membranes of granules digested for 14 h; lane 7, membranes of granules digested for 24 h; lane 8, granule membranes.

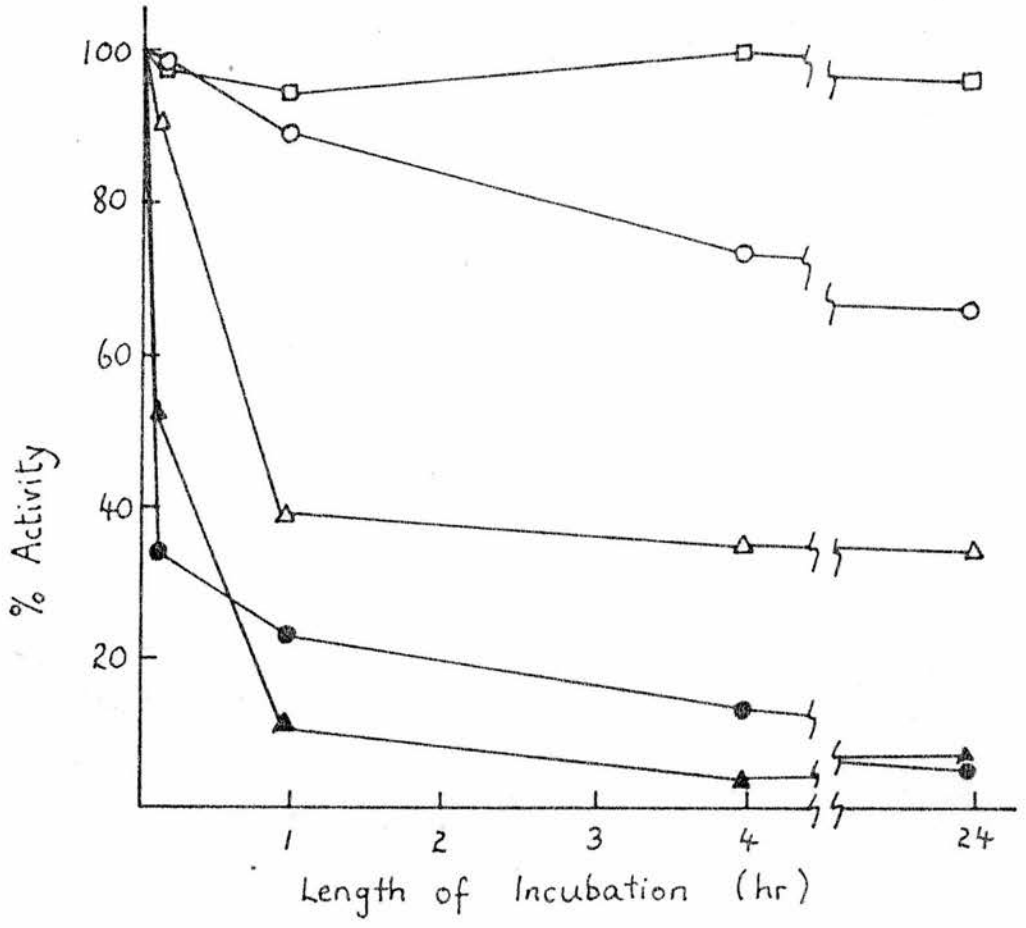


Fig. 5.6 Effect of Pronase Digestion of Granule on Membrane Bound Enzyme Activities

Chromaffin granules were digested with 0.1 mg/ml pronase for 0 to 24 h at room temperature. Membranes prepared from the digested granules were assayed for dopamine-β-hydroxylase (-□-), NADH: (acceptor) oxidoreductase (-△-), Mg^{++} ATPase (-●-), and phosphatidylinositol kinase (-▲-). Enzyme activities were expressed as the amount of substrate converted per mg cholesterol per min at the assay temperature. The course of the digestion is indicated by the fall in the protein to cholesterol ratio in the membrane (-○-). "Control" membranes were prepared from granules incubated at room temperature for 4 h (for 10 min, 1 h and 4 h time points) and 24 h (for 24 time point).

kinase, and NADH: (acceptor) oxidoreductase. The progress of the digestion is indicated by the fall in the protein to cholesterol ratio of the membrane and all enzyme activities are expressed in terms of the amount of cholesterol present in the membrane samples.

Membrane-bound dopamine-β-hydroxylase is unaffected by pronase treatment of intact granules, 96% of its activity remaining after 24 hr digestion which had resulted in lysis of 50% of the granules (Table 5.1). The original activity of DβH in the membrane was 12.5 nmole tyramine converted per mg cholesterol per min at 37°C, equivalent to 13.3 nmoles tyramine converted per mg protein in untreated granule membranes.

Mg^{++} ATPase, phosphatidylinositol kinase, and NADH: (acceptor) oxidoreductase all show progressive decrease in activity with increasing length of incubation of the granules with 0.1 mg/ml pronase. These three enzymes can all be reduced in activity by at least 75%, phosphatidylinositol kinase being the most susceptible to pronase digestion of the granule followed by the Mg^{++} ATPase and finally the NADH:(acceptor) oxidoreductase. The activities of these enzymes in membranes prepared from granules incubated at room temperature in the absence of pronase were 0.5 nmole phosphate incorporated per mg cholesterol per min at 37°C (equivalent to 0.54 nmole phosphate incorporated per mg protein) for phosphatidylinositol kinase, 52.9 nmole phosphate released per mg cholesterol per min at 37°C (equivalent to 56.4 nmole phosphate released per mg protein) for the Mg^{++} ATPase, and 0.11 μmole NADH oxidized per mg cholesterol per min at room temperature (equivalent to 0.12 μmole NADH oxidized per mg protein) for the NADH:(acceptor) oxidoreductase.

5.3.1.5 Effect on Cytochrome b-561

The oxidized versus reduced spectrum of granule membranes prepared from granules digested for 4 hr at room temperature with 0.1 mg/ml pro-

nase was the same as that previously reported for undigested granule membranes over the range 390 - 700 nm (see section 3.3.8). Membrane samples prepared from granules incubated at room temperature with or without pronase showed a reduced versus oxidized absorption peak of 0.106 absorbance units per mg cholesterol at 561 nm.

5.3.2 Digestion of Isolated Granule Membranes

5.3.2.1 Effect on Protein Content

Isolated granule membranes (1 mg/ml protein) were digested with 0.1 mg/ml pronase at room temperature for varying periods of time. 70% of the protein content of the membrane was removed after only 10 min digestion. However, no further loss in protein occurred after 1 and 4 hr incubation with pronase. Digestion would appear to be complete after 10 min under these conditions.

5.3.2.2 Effect on CBB Staining Profile

Fig. 5.7 shows a photograph of a CBB stained gel containing granule membranes digested with 0.1 mg/ml pronase at room temperature for 10 min, 1 hr, and 4 hr together with a sample of undigested granule membranes. All the changes in the CBB staining profile of the membrane occur after only 10 min digestion with pronase and these changes are more extensive than when intact granules are digested. The major staining component of granule membranes, dopamine-β-hydroxylase, has been degraded. A broad band (K), of apparent molecular weight 59,000 daltons, appears and is probably a digested form of dopamine-β-hydroxylase. Band K is relatively poorly stained, however, so it probably undergoes further digestion. Chromomembrin B and band G and H, the components to which chromomembrin B is degraded when intact granules are digested, are all missing when granule membranes are digested with pronase.

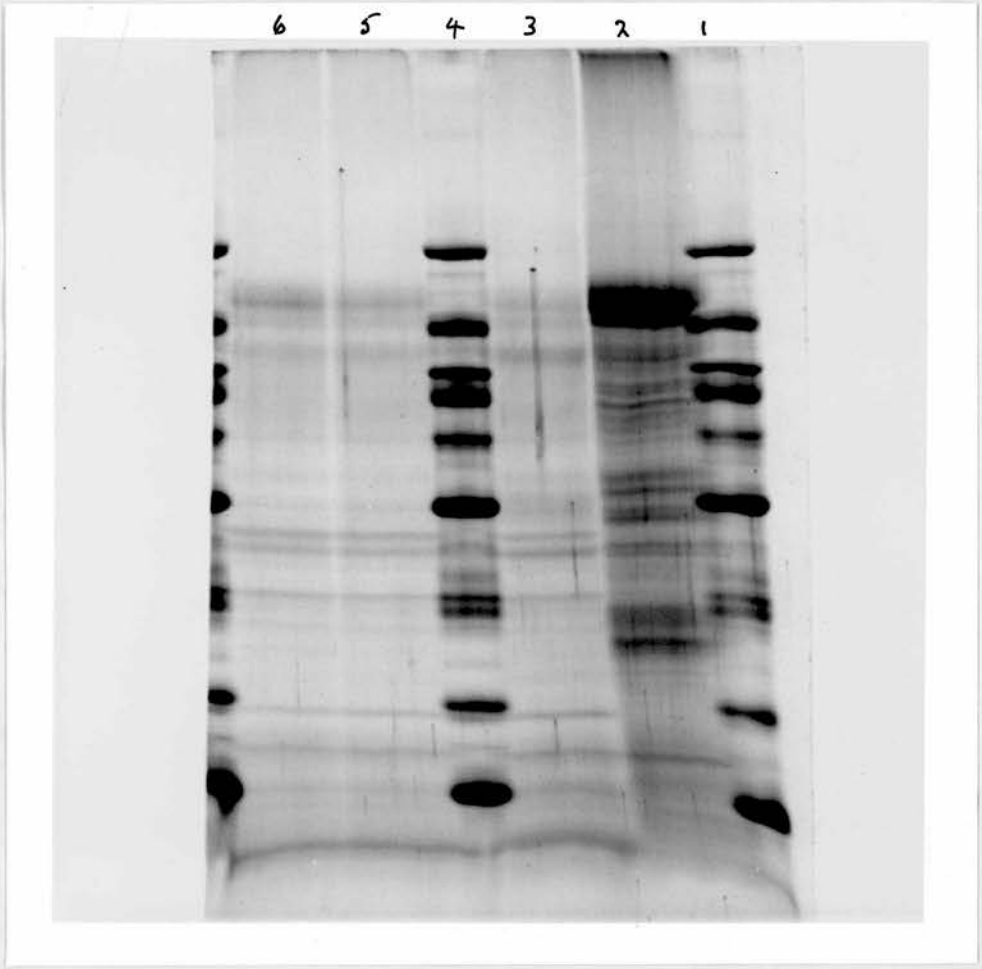


Fig. 5.7 Pronase Digestion of Purified Chromaffin Granule Membranes - Effect on CBB Staining Gel Pattern

Isolated granule membranes were digested for 0 to 4 h with 0.1 mg/ml pronase at room temperature. After washing, the membranes were subjected to SDS polyacrylamide gel electrophoresis in 8% - 20% (w/v) acrylamide gradient gels under reducing conditions. The gel was then stained with coomassie brilliant blue. Lanes 1 and 4, molecular weight markers (see Fig. 5.1); lane 2, control membranes prepared from membranes incubated at room temperature for 4 h; lanes 3, 5 and 6, membranes digested with pronase for 10 min, 1 h and 4 h respectively. Equal amounts of membrane (0.1 mg choesterol) were applied to each lane.

Although faint traces of many CBB stained bands remain only two bands, 61 and 64, are apparently totally unaffected by pronase digestion of the membranes. Thus CBB stained bands 13, 14, 15, 16, 17, 25 and 27, which were resistant to digestion in granules, were all degraded. However, degradation bands D, E and F, which occur following pronase treatment of granules, are also present in pronase treated granule membranes together with the degradation bands K,L,M,N and O exclusive to pronase digested granule membranes.

5.3.2.3. Effect on Carbohydrate Staining Profile

In addition to PAS band Ib (see section 5.3.1.3), PAS bands II (dopamine-β-hydroxylase) and V (GP VII) are missing from the PAS staining profile of isolated granule membranes digested with pronase (Fig 5.8) No new PAS staining bands are observed. PAS bands Ia, III and IV are resistant to proteolysis in granules and isolated membranes.

5.3.2.4. Effect on Enzyme Activities

Dopamine-β-hydroxylase could be inhibited by pronase digestion (0.1 mg/ml) of isolated granule membranes, 75% of its activity being lost following 10 min exposure at room temperature of the membranes to pronase. However, longer incubation of the membranes with pronase (1 and 4 hr) failed to inactivate the enzyme any further. The Mg^{++} ATPase and NADH:(acceptor) oxidoreductase of the granule membrane also showed residual activities following digestion of the membrane. 80% of the activity of the Mg^{++} ATPase and 70% of that of the NADH:(acceptor) oxidoreductase was lost after 10 min digestion but no further reduction occurred after 1 hr and 4 hr digestions. The original enzyme activities of these membranes were 6.2 nmole tyramine converted per mg cholesterol per min at 37°C for dopamine-β-hydroxylase, 53.1 nmole phosphate released

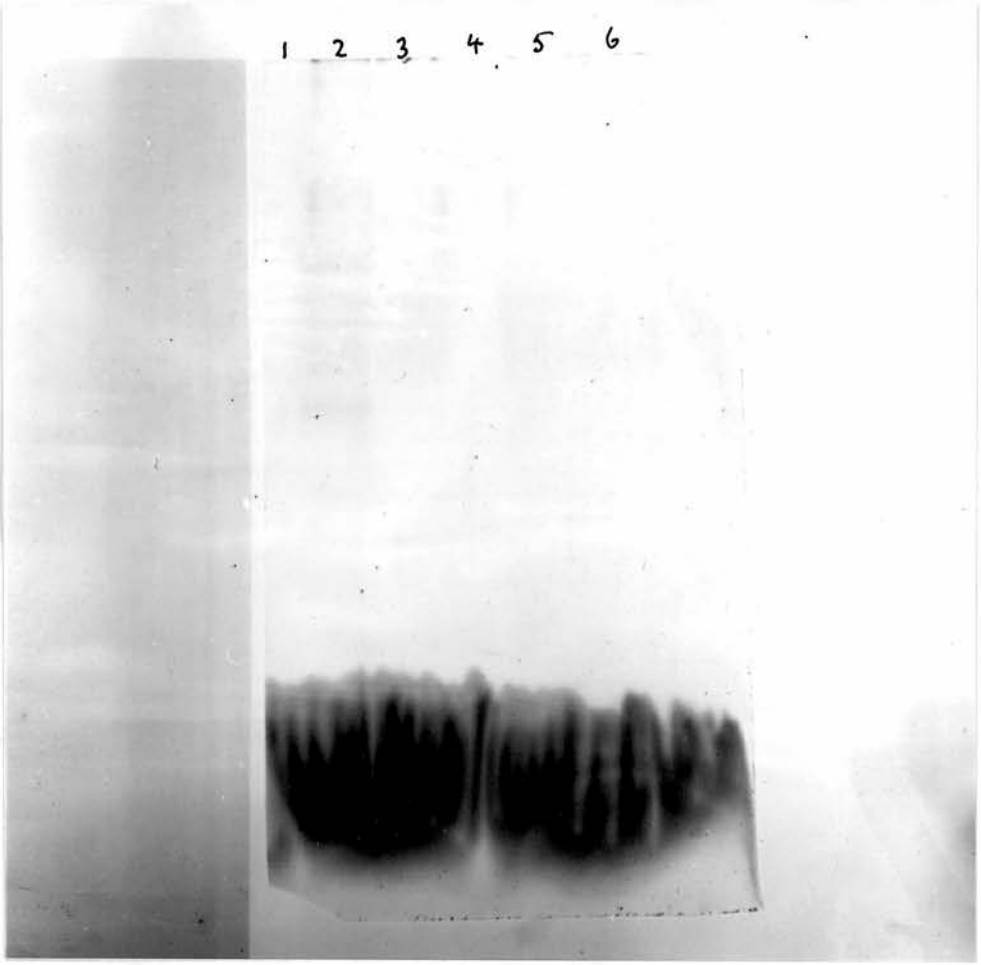


Fig. 5.8 Pronase Digestion of Purified Chromaffin Granule Membranes - Effect on PAS Staining Gel Pattern

A duplicate gel to the one shown in Fig. 5.7 was stained for carbohydrate by the periodic acid - Schiff procedure. Lanes 1 and 4, molecular weight markers; lane 2, control membranes incubated for 4 h at room temperature in the absence of pronase; lanes 3, 5 and 6, membranes digested with pronase for 10 min, 1 h and 4 h respectively.

per mg cholesterol per min at 37°C for the Mg^{++} ATPase, and 0.146 μmole NADH oxidized per mg cholesterol per min at room temperature.

5.3.2.5 Effect on Cytochrome b-561

Cytochrome b-561 reduced versus oxidized absorption peak is unaffected by extensive digestion of granule membranes with pronase (0.1 mg/ml for 4 hr at room temperature) as is the rest of the reduced versus oxidized spectrum. Pronase digested membranes reduced with dithionite showed an absorption of 0.116 absorption units per mg cholesterol at 561 nm relative to non-reduced pronase digested membranes. For membranes incubated in the absence of pronase this parameter was 0.125 absorption units.

5.4 Discussion

5.4.1. The Use of Pronase to Investigate the Arrangement of Proteins in the Chromaffin Granule Membrane

The water-soluble proteases of Streptomyces griseus, available as the commercial preparation known as pronase, have been used to digest intact chromaffin granules and isolated granule membranes. The coomassie brilliant blue and periodic acid - Schiff staining patterns of gels of the various digested membranes as well as the activities of dopamine-β-hydroxylase, Mg^{++} activated ATPase, phosphatidylinositol kinase and NADH:Acceptor oxidoreductase and the reduced versus oxidized spectrum of the digested membranes were then compared with those of undigested chromaffin granule membranes. Because of the removal of protein from the membrane by pronase membranes were quantified in terms of their cholesterol content.

The use of pronase to study the arrangement of the membrane proteins of the chromaffin granule has several advantages compared to the use of other labelling techniques. The majority of small molecular weight so-called impenetrant chemical labelling reagents possess a small but nevertheless finite membrane permeability whereas proteases are totally membrane impermeable since it is their large size which is responsible for this impenetrability. Labelling of chromaffin granule membrane proteins is often very poor because of labelling of catecholamines and/or phospholipids. This is observed with both the use of chemical labelling reagents, e.g. diazobenzene [^{35}S]sulphonate (Konig et al., 1976) and enzymic labelling techniques, e.g. lactoperoxidase catalyzed radioiodination (see chapter 6). Catecholamines and phospholipids will not interfere with proteolytic digestion. Furthermore no time consuming or sophisticated techniques such as fluorography, autoradiography, gel slicing, and fluorescence scanning are required to detect which components are sensitive to pronase digestion. Proteins degraded by pronase will have a smaller molecular weight and hence faster mobility upon SDS polyacrylamide gel electrophoresis (Shapiro et al., 1967; Weber and Osborn, 1969; Lambin, 1978) compared to the native protein. Pronase digestion usually has a damaging effect upon enzymes which are exposed to the proteases and hence the effect of digestion of granules and isolated membranes can also be followed by the assay of the membrane bound enzymes.

Because of the extensive damage which proteolysis produces lysis of cells or organelles is often a serious problem. Lysed granules may be digested with pronase at both sides of the membrane and therefore care must be taken either to limit lysis by the use of milder digestion conditions or to purify only intact granules. The sedimentation of digested

granules through 1.8 M sucrose will result in the removal of lysed granules as well as other contaminating organelles. Therefore since we can separate intact and damaged granules we can use harsher conditions to digest the granules in order to seek out those components which are accessible to pronase but are more resistant to digestion. This may help to increase the sensitivity of detecting components exposed on the outside of the granule since Bender et al. (1971) noted that the diazonium salt of sulphanilic acid labels more proteins in the intact erythrocyte than are digested with pronase. Presumably the diazonium salt of sulphanilic acid is less sterically hindered than proteases.

Another disadvantage concerning the use of proteases is the ability of sodium dodecyl sulphate not only to denature membrane proteins but also to heighten their sensitivity to contaminating proteases (Bender et al., 1971; Fairbanks et al., 1971; Steck et al., 1971). To try and eliminate this problem digested membranes were solubilized in pre-heated sodium dodecyl sulphate at pH 4 as recommended by Bender et al. (1971) and high concentrations of pronase were avoided, the extent of digestion being controlled by the length of incubation with a fixed low concentration (0.1 mg/ml) of pronase. For granules we can conclude that even if proteolysis does occur during sodium dodecyl sulphate solubilization prior to electrophoresis it only occurs to a minor extent compared to proteolysis occurring during the incubation periods since several proteins, including DβH are resistant to pronase digestion in granules even though they are sensitive to digestion when isolated membranes are treated. Furthermore there are substantial differences in the patterns of digestion of granules incubated with a fixed concentration of pronase for 10 minutes, 1 hr and 4 hr. These differences can only have been due to the increased length of exposure of the granules to pronase since all

other parameters such as the concentration of pronase and hence the level of contaminating pronase present during SDS solubilization were the same.

When isolated granule membranes are digested with 0.1 mg/ml pronase at room temperature no differences are observed between the CBB or PAS stained gels of membranes digested for 10 min 1 hr or 4 hr. Furthermore few CBB stainable components are resistant to pronase digestion. Therefore it would seem difficult to decide whether the digestion occurring has happened during the incubation period or SDS solubilization prior to electrophoresis. However, three of the five PAS staining regions of gels of granule membranes are resistant to pronase digestion and enzyme assays and protein estimation indicate that digestion is complete within 10 minutes. Also residual traces of many CBB stainable components and of enzyme activities are present. Therefore, I would tentatively conclude that when isolated granule membranes are digested with pronase and subjected to SDS polyacrylamide gel electrophoresis the pattern of digestion observed is mainly due to that occurring during the incubation period with pronase rather than during SDS solubilization.

5.4.2 The Effect of Pronase on the CBB Staining Pattern of Gels of Granule Membranes

The use of slab gels, in which several samples can be electrophoresised in the same gel under identical conditions, has allowed detailed comparisons to be made between digested and control granule membrane samples. The same polypeptides present in different samples will migrate the same distance and will be located side by side (unless it has been degraded). Furthermore by applying the same amounts of membrane to the gel (in terms of cholesterol content) and using the

knowledge that, under identical conditions of CBB staining, the amount of CBB bound by a particular polypeptide is proportional to the amount of protein present (Fairbanks et al., 1971) we can identify partially digested polypeptides by their reduction in staining intensity.

The use of SDS polyacrylamide gel electrophoresis to identify pronase sensitive polypeptides has a number of drawbacks which must be borne in mind when interpretating the results of experiments. CBB stained bands may contain more than one polypeptide and this may explain why some bands show an initial decrease in CBB staining intensity but remain resistant to further digestion. Although bands which do not show a change in mobility are considered resistant to pronase they may actually have been degraded. Changes of 2 - 3% or less in the molecular weight of a polypeptide will not appreciably affect its mobility. The appearance of new CBB stained bands lower down the gel, due to the degradation of larger molecular weight components, may obscure regions of the gel pattern and may also be confused with native bands. Apparent reductions in staining intensities of minor CBB staining components may be due to the removal of a closely migrating major staining component.

Of the 66 CBB stained bands identified in SDS gels of reduced granule membranes 38 are sensitive to pronase degradation in intact granules. 28 of these bands (CBB stained bands 1,2,3,4,5,6,18,19,20,23, 26,34,35,41,42,45,47,48,49,50,51,53,54,56,57,59,60 and 56) are moreorless completely digested whilst the remaining 10 bands (CBB stained bands 22,24,28,29,30,31,32,37,38 and 39) show a marked reduction in staining intensity. Only ten bands were identified as being resistant to pronase in intact granules (CBB stained bands 13,14,15,16,171,21,25,27,61 and 64).

The remaining 18 CBB stained bands could not be assigned as being sensitive or resistant either because of the appearance of new staining bands obscuring their position in the gel or because of uncertain identification of the band, particularly in the case of minor staining components.

Dopamine-β-hydroxylase has previously been identified as CBB staining bands 13, 14 and 15 of gels of reduced granule membranes (see section 3.3.4.) and is clearly resistant to pronase digestion in intact granules. On the other hand chromomembrin B the second major staining region of the gel (CBB stained bands 49,50 and 51) is sensitive giving rise to, firstly, the degraded component G and, then, to H (see Fig. 5.1 and 5.2). Eight other new bands were observed (CBB stained bands A, B,C,D,E,F,I,J) when granules were treated with pronase but none of these degradation products could be identified with the band from which they had arisen. CBB stained bands 28, 29 and 44 which are thought to contain the subunits of the Mg^{++} ATPase show only a partial reduction in staining intensity in gels of membranes prepared from digested granules. It is impossible to decide whether this reduction in staining is due to the ATPase subunits or to other polypeptides which co-migrate, with the ATPase subunits. No other CBB stained bands of gels of reduced granule membranes have been unequivocably identified.

When isolated granule membranes are treated with pronase in addition to the 38 bands degraded when granules are digested CBB stained bands 13, 14, 15, 16, 17, 25 and 27 are removed from the profile. These bands, which include those representing DβH, are therefore probably exclusively located on the inner surface of the granule membrane. Band 21 which is resistant to digestion in granules is obscured by the breakdown product of bands 13, 14 and 15 when isolated membranes are digested whilst bands 61 and 64 are completely resistant to pronase digestion of intact granules and purified membranes and thus may be completely buried

in the membrane bilayer. CBB stained bands G and H which are derived from the digestion of chromomembrin B in intact granules have been further degraded in which case chromomembrin B is likely to be exposed at both surfaces of the granule membrane. Other polypeptides are also digestable from both sides of the membrane since bands B,C,I and J which are digestion products of polypeptides digested in intact granules are also degraded in isolated membranes. Bands D,E and F occur in gels of membranes prepared from both digested granules and membranes and therefore the native polypeptide from which they are derived are located only in the outer surface of the membrane. New bands K (derived from Dβ H), L,M,N and O appear only in gels of pronase digested isolated membranes and therefore must have arisen from bands 13,14,15,16,17,25 and 27.

Thus nearly all the polypeptides of the chromaffin granule membrane are exposed at one or the other or both surfaces of the membrane with the majority being exposed at the outer surface of the granule. Dopamine-β-hydroxylase is exposed only at the inner surface of the membrane facing the matrix of the granule whilst chromomembrin B is exposed at both faces of the membrane and may be transmembraneous.

5.4.3 The Effect of Pronase on the PAS Staining Profile of Gels of Granule Membranes

Interpretations of the effect of pronase on the PAS staining profile of gels of granule membranes are subject to the same constraints as those listed for the effects of pronase on the CBB staining profile of the membranes. In addition because of the diffuse nature of the PAS stain it would be difficult to detect changes in the intensity of staining.

The five PAS staining regions of gels of reduced granule membranes were identified in membranes prepared from pronase digested granules

although the leading edge of PAS band I was missing. Thus at least one glycoprotein is exposed at the outer surface of the chromaffin granule. [^{3}H]borohydride labelling following galactose oxidase treatment (see chapter 4) indicates that all the carbohydrate chains of the granule membrane glycoproteins face the matrix of the granule. Therefore GP Ib may be exposed at both surfaces of the granule membrane. PAS bands II and V are missing when isolated granule membranes are digested with pronase. Therefore GP II (DβH) and VII which are represented by these PAS staining bands (see chapter 4) are exposed at the inner surface of the granule membrane. The assignment of DβH to the inner surface of the membrane is in agreement with the effect of pronase on the CBB staining and enzyme activity of membrane bound DβH.

Huber et al. (1979) and myself (chapter 4) have shown that the carbohydrate residues of the major glycoproteins are exposed only at the inner surface of the membrane. Therefore, failure of GPs Ib, IV, V and VI to be degraded by pronase in isolated membranes may indicate steric hindrance of pronase by the carbohydrate moieties of these glycoproteins. Alternatively the peptide portions of these glycoproteins may not contain pronase susceptible bands or the peptide backbone may be buried in the membranes.

We may also conclude that the CBB staining bands which co-migrate with GPs Ia, IV,V,VI and VII do not actually represent these glycoproteins. In granules the majority of these CBB staining bands are digested whereas only GP Ib is susceptible. It does seem likely,however, that GP VII is represented by the broad residual staining remaining after digestion of CBB stained bands 37, 38 and 39.

Because of the failure to digest GPs Ia,IV, V and VI under any conditions this technique does not allow one to hazard a guess as to their

location in the granule membrane. Furthermore pronase digestion does not yield information regarding the location of the carbohydrate moieties of the glycoproteins. This is fully discussed in chapter 4.

5.4.4 The Effect of Pronase on Membrane Bound Enzyme Activities of the Granule

Dopamine-β-hydroxylase activity is totally resistant to pronase digestion in intact granules. However, three quarters of its activity is lost within 10 minutes digestion of isolated granule membranes with pronase. It may be that a small portion of DβH is exposed on the outside of the granule and this may be removed without loss of activity. (It is known that certain membrane bound enzymes can be solubilized by proteases which cleave the major part of the protein including the active site from the membrane leaving behind that part of the peptide which anchors the enzyme to the membrane. The anchor region is not required for enzyme activity - see Kenny and Booth, 1979). This region would have to be small for it not to affect the mobility of DβH and it clearly does not contain the carbohydrate region of DβH. Thus the likeliest explanation is that DβH is localized exclusively at the inner surface of the granule membrane.

Kirshner (1962) drawing conclusions from studies of inhibitors of dopamine uptake into chromaffin granules suggested that the conversion of dopamine to noradrenaline occurred within the granule indicating that at least the active site of DβH, the enzyme responsible for this conversion, is located inside the granule. A similar conclusion can be made from the experiments of Laduron (1975) on the latency of DβH in chromaffin granules. Konig, et al. (1976) using diazobenzene [^{35}S]sulphonate, an impenetrant chemical labelling reagent showed that DβH could only be

significantly labelled if isolated membranes were used. Microcomplement fixation has also been used to localise DβH to the inner surface of the granule membrane (Konig et al., 1976) although a small amount of microcomplement fixation occurred with intact granules. This was suggested as being due to contaminating membrane fragments and soluble DβH (Konig et al., 1976). Thomas et al. (1973) demonstrated some inhibition of the enzyme after treatment of intact granules with an antiserum but Konig et al. (1976) suggest that their results can also be interpreted in terms of contamination with soluble DβH and membrane fragments. The carbohydrate region of DβH has also been shown to face the inside (Huber et al., 1979) whilst this conclusion can be inferred from the findings of Eagles et al. (1975) who showed that con A binding sites are located only on the inner surface of the membrane and Rush et al. (1974) who found that DβH binds very effectively to con A.

The other three membrane bound enzymes, Mg^{++} activated ATPase, phosphatidylinositol kinase and NADH: Acceptor oxidoreductase can all be inhibited in intact chromaffin granules with pronase. These enzymes are generally considered to have substrate binding sites located on the outer surface of the membrane in view of the hydrophilic nature of their substrates. The accessibility of these enzymes to pronase is thus not unexpected. Only the NADH:acceptor oxidoreductase showed appreciable residual activity (25%) after digestion of the granules for 24 hr with 0.1 mg/ml pronase. This may be due to an alteration in the catalytic properties of the enzyme by proteolysis rather than total inactivation.

Residual activities were also observed however, when isolated membranes were digested with pronase. This applied to DβH and the Mg^{++} ATPase as well as the NADH:acceptor oxidoreductase, residual activities being 25%, 20% and 30% respectively. The most likely explanation in this

case is that the membrane preparations used for digestion contained resealed vesicles (both 'inside out' and right-side out') as well as broken membranes so that some active sites were protected from pronase access. These would be revealed during assay owing to subsequent freezing and thawing of the membranes. Traces of most of the major CBB staining components in gels of membranes digested after isolation which fail to disappear after further digestion of the membranes also suggest that vesiculation has occurred prior to the addition of pronase. Eagles et al. (1975) have shown that negative staining of granule membranes show opaque sacs indicating that the membranes have resealed and have prevented the stain from penetrating.

5.4.5 The Effect of Pronase on the Reduced versus Oxidized Spectrum of Granule Membranes

The presence of an absorption maximum at 561nm in the reduced spectrum of granule membranes has been attributed to the presence of a b type cytochrome in the granule membranes (see section 3.3.8). This absorption peak is unaffected by both digestion of intact granules and isolated granule membranes with pronase. The cytochrome b-561 may not even be intrinscially resistant to pronase digestion since the absorption peak at 561 nm indicates only that the haem group of the cytochrome is retained within the membrane. Thus one cannot draw any conclusions whatsoever regarding the location of cytochrome b-561 in the chromaffin granule membrane.

CHAPTER 6

LACTOPEROXIDASE CATALYZED IODINATION OF CHROMAFFIN GRANULES

LACTOPEROXIDASE CATALYZED IODINATION OF CHROMAFFIN GRANULES

6.1 Introduction

The peroxidase from milk, lactoperoxidase (E.C.1.11.1.7),has been purified (Theorell and Akeson, 1943; Polis and Shmukler, 1953;Morrison, 1970; Morrison and Hultquist, 1963) and shown to be capable of iodinating phenolic compounds including tyrosine (and possibly histidine) residues of polypeptides and proteins (Marchalonis, 1969). The reaction is hydrogen peroxide dependent and occurs via an enzyme substrate complex involving the compound to be iodinated (Morrison and Bayse, 1970). Other peroxidases will also iodinate phenolic compounds but only indirectly since they catalytically oxidize iodide to iodine which, at neutral and high pHs, reacts spontaneously with phenolic compounds (Mayberry et al., 1964; Thomas and Hager, 1969).

Phillips and Morrison (1969) suggested a technique for examining the spatial arrangement of proteins in membranes using the lactoperoxidase catalyzed, hydrogen peroxide dependent iodination technique. They reasoned that, because of its size, 78,000 daltons (Rombauts et al., 1967), preventing it from crossing membranes and the need for the iodinatable substrate to be directly accessible to the enzyme, lactoperoxidase would be capable of iodinating only those membrane proteins exposed on the outer surface of intact cells or organelles. Comparison of the labelling pattern of isolated membranes with that of membranes prepared from labelled intact cells or organelles should allow identification of membrane proteins exposed at the interior of the cell or organelle.

During lactoperoxidase catalyzed iodination of cells or organelles the concentration of hydrogen peroxide must be kept low to prevent lipid peroxidation which is known to affect the permeability properties of

membranes (Welton and Aust, 1972; Morrison, 1974). Phillips and Morrison (1971a) kept the concentration of hydrogen peroxide below 8 μM by the addition of small aliquots of hydrogen peroxide at fixed intervals of time while Reichstein and Blostein (1973) used glucose and glucose oxidase to generate hydrogen peroxide at a continuous low concentration. Alternatively Welton and Aust (1972) have used the antioxidant butylated hydroxytoluene to prevent lipid peroxidation. Butylated hydroxytoluene also prevents loss of cytochrome p-450 from the endoplasmic reticulum and increases incorporation of iodide into these membranes. (Welton and Aust, 1972).

Iodide in gels of iodinated membrane proteins separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis can easily be detected, if ^{125}I or ^{131}I are used, by autoradiography or by estimating radioactivity in gel slices by gamma spectrometry or liquid scintillation counting.

Since its introduction the lactoperoxidase labelling technique has been used to investigate the spatial arrangement of membrane proteins in plasma membranes (the erythroycte, normal and transformed cells etc), intracellular membranes (inner and outer mitochondrial membranes, endoplasmic reticulum, sarcoplasmic reticulum, golgi apparatus, and lysosomal membrane) and viruses (see Carraway, 1975, for review). The versatility of this method is illustrated in studies with the erythrocyte membrane. Intact whole cells (Phillips and Morrison, 1970, 1971a) isolated membrane ghosts (Marchesi et al., 1972), and resealed inside out (Reichstein and Blostein, 1975) and right side out membrane vesicles have been iodinated. In addition lactoperoxidase can be sealed inside membrane vesicles (Shin and Carraway, 1974; Reichstein and Blostein, 1973; Mueller and Morrison, 1974) such that only the inner surface of

the membrane is iodinated since hydrogen peroxide and iodide are membrane permeable. Proteins exposed at both membrane surfaces can be identified by firstly maximally iodinating whole intact erythrocytes with ^{125}I, preparing membrane ghosts, and then labelling newly accessible sites with ^{131}I.

This chapter reports on the labelling of intact chromaffin granules, isolated granule membranes, and resealed membrane ghosts by the lactoperoxidase catalyzed, hydrogen peroxide dependent labelling technique using ^{125}I. The iodinated membranes are subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis. Protein is detected by coomassie brilliant blue staining and ^{125}I by gel slicing and gamma spectrometry or autoradiography of dried gels. Problems associated with labelling of chromaffin granules by this procedure are discussed as well as general problems concerned with the use of lactoperoxidase catalyzed iodination.

6.2 Materials and Methods

6.2.1 Materials

^{125}I (carrier free, NaI in NaOH) was obtained from the Radiochemical Centre, Amersham, U.K.; butylated hydroxytoluene from Koch-Light Laboratories Ltd., Colnbrook, Bucks, U.K; Analar hydrogen peroxide (30%, w/v) from BDH, Ltd., Poole, Dorset, U.K. and lactoperoxidase from Boehringer.

The specific activity of lactoperoxidase was 160 μ/mg when measured using a hydrogen peroxide, 2.2'-azino-di[3-ethyl-benzthiazoline sulphonate (6)]-diammonium salt (ABTS) assay system and came as a 5 mg/ml suspension in 3.2 M ammonium sulphate, pH 7.

6.2.2 Iodination of Intact Chromaffin Granules

P_2 chromaffin granules were centrifuged at 18,000 g-av, for 20 min at 2°C. The pellet was gently washed with 0.3 M sucrose, 10 mM hepes, pH 7 before being resuspended in an equal volume of this buffer. To 0.5 ml of the resuspended granules was added 0.8 μ lactoperoxidase, 200 μCi Na ^{125}I and 1 μl 2 mg/ml butylated hydroxytoluene in ethanol. The final concentration of ethanol was 0.2%. The granules were then equilibrated at 12 - 15°C before iodination was initiated by the addition of 2 μl 100 mM H_2O_2 in 0.3 M sucrose, 10 mM hepes, pH 7. Further 2 μl aliquots of H_2O_2 were added every 4 min for 1 hr giving a total addition of 3 μmoles of H_2O_2. The reaction was terminated by transferring the granules to ice and adding 1 μl β-mercaptoethanol (14.3 M) before granule purification and membrane preparation (section 2.1.2). Memberanes were washed with 10 mM hepes, 10 mM KI, pH 7. The chromaffin granule lysate was also retained.

6.2.3 Iodination of Isolated Chromaffin Granule Membranes

2 ml freshly prepared or freeze thawed granule membranes suspended in 10 mM hepes, pH 7 at 1 mg/ml protein were iodinated at R.T. with 200 μCi Na ^{125}I, 0.08 μ lactoperoxidase, and 0.75 μmoles hydrogen peroxide added in 15 5 μl aliquots of 10 mM H_2O_2 in 10 mM hepes, pH 7 at 4 min intervals. 2 μl of a 2 mg/ml solution of butylated hydroxytoluene in ethanol was also included, giving a concentration of 0.1% ethanol in the incubation mix. The reaction was stopped with 1 μl β-mercaptoethanol (14.3 M) and cooling to 0°C. Iodinated membranes were washed three times with ice cold 10 mM hepes, pH 7, 10 mM KI.

Granule membranes treated with 0.1% triton X-100 were also iodinated in the manner described above. Excess ^{125}I was removed by dialysis against 0.1% triton X-100, 10 mM KI, 10 mM hepes, pH 7 at 4°C.

6.2.4 Iodination of Total Chromaffin Granule Lysate

Granule lysate (1 ml, 2 mg/ml protein), prepared as described in section 2.1.2, was iodinated in the same manner as intact granules except that the reaction was carried out at room temperature. The iodinated lysate was then passed down a small sephadex G-25 column equilibrated in 10 mM hepes, pH 7 and the excluded volume retained.

6.2.5 Iodination of Resealed Chromaffin Granule Membrane Ghosts

Resealed membrane ghosts (1 ml, 0.5 mg/ml protein), prepared according to Phillips (1974a, 1977), were iodinated at 12 - 15^{o}C as described for isolated granule membranes. The iodinated ghosts were pelleted by centrifugation at 100,000 g-av for 1 hr at 2^{o}C and then ruptured by hypotonic shocking followed by washing with 10 mM KI, 10 mM hepes, pH 7. Membranes were recovered by centrifugation (160,000 g-av for 1 hr at 2^{o}C). Hypotonic shocking, washing and centrifugation were repeated before final resuspension in 10 mM hepes, pH 7.

6.2.6 Dichloromethane Extraction of Chromaffin Granule Membranes

Granule membranes prepared from iodinated granules were dichloromethane extracted according to the method of Apps and Glover (1978). The extracted membranes were then concentrated by freeze-drying.

6.2.7 Detection of ^{125}I in Gels by Gamma Spectrometry

Individual lanes of slab gels were sliced transversely into clmm thick sections using a series of razor blades separated by washers. The apparatus was placed on top of the gel, itself placed on a flat surface, hand pressure applied and the gel slices flicked from between the blades into scintillation vial insets. The samples were then counted directly in a gamma spectrometer using the following settings: fine gain, 1.3; window, 40; threshold, 40.

6.2.8 Autoradiography

Gels were dried down between two sheets of porous cellophane using the gel drying apparatus supplied by Raven Scientific, Limited. X-ray film was then exposed to the gel and, after a suitable interval of time, developed with Universal film developer (1 part per 20 parts water) and fixed with potassium thiosulphate.

6.3 Results

6.3.1 Iodination of Intact Chromaffin Granules

6.3.1.1 Incorporation of ^{125}I into the Granule Membrane

Fig. 6.1. shows the distribution of radioactivity in an 8% acrylamide gel containing granule membrane proteins separated by SDS polyacrylamide gel electrophoresis under reducing conditions. This radioactivity profile was determined by slicing the gel into uniform sections and counting the pieces in a gamma spectrometer. Granule membranes were prepared from intact granules labelled with ^{125}I by the lactoperoxidase procedure. Also shown in Fig. 6.1 is the CBB staining profile of the gel which is similar to that of gels of reduced granule membranes previously described (see section 3.3.1).

The radioactive and CBB staining profiles of the gel of reduced granule membranes shown in Fig. 6.1 have been compared in Table 6.1, column one by assigning between one and five pluses to each CBB stained band according to the amount of radioactivity associated with its position in the gel. The results of three other experiments in which granule membranes were radioiodinated by lactoperoxidase in the granule state are also summarized in Table 6.1 (columns 2,3 and 4).

Although there is a variable level of

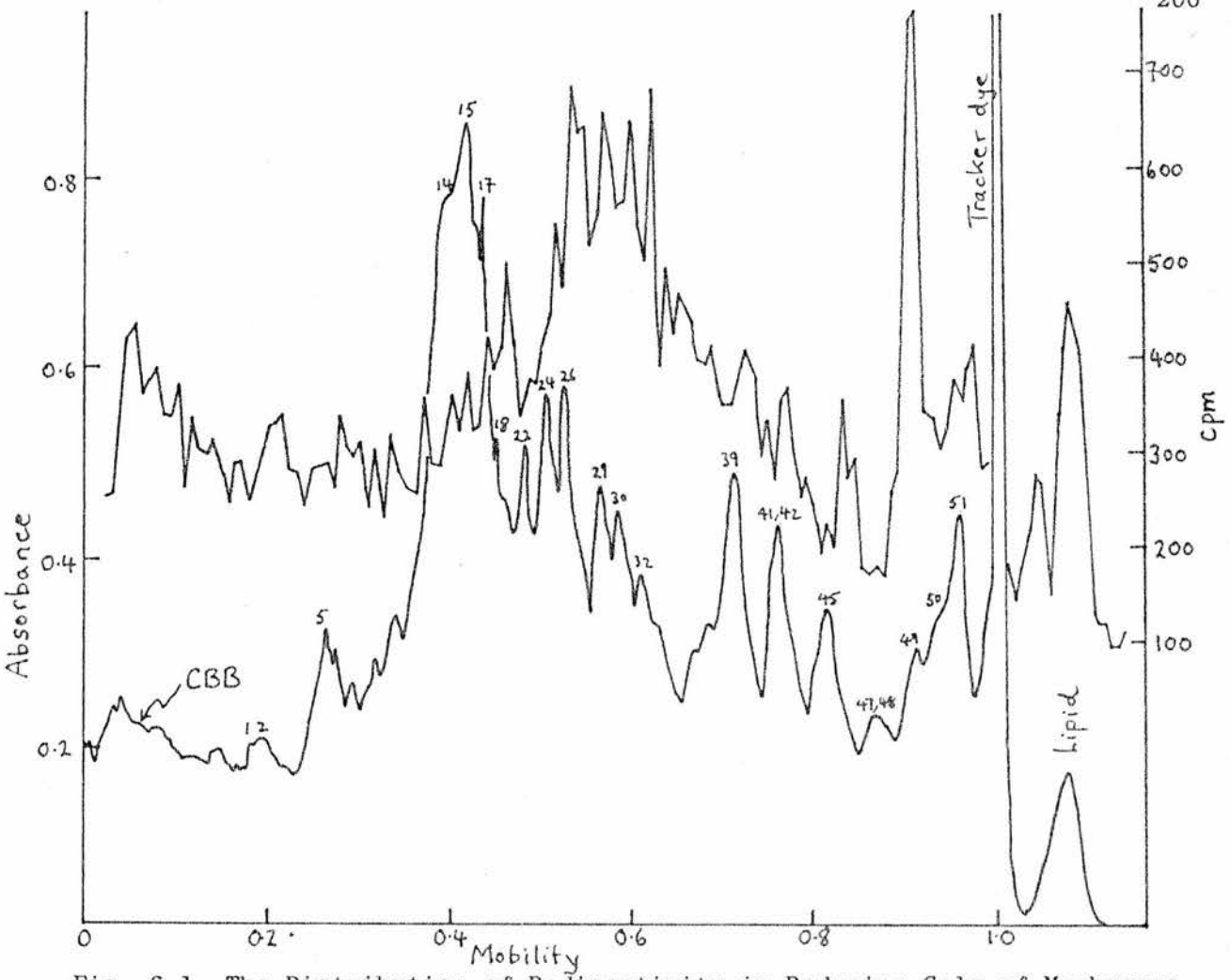


Fig. 6.1 The Distribution of Radioactivity in Reducing Gels of Membranes of Lactoperoxidase Iodinated Chromaffin Granules

Membranes prepared from iodinated granules were separated by SDS polyacrylamide gel electrophoresis under reducing conditions in 8% acrylamide gels. After staining for protein with CBB and densitometric scanning at 570 nm, the distribution of radioactivity in the gel was determined by gamma spectrometry of 1 mm sections of the gel. Crude granules (0.5 ml, 15-20 mg/ml protein) in 0.3 M sucrose, 10 mM hepes, pH 7 were iodinated at 12 - 15°C with 0.8 units lactoperoxidase, 200 μCi Na ^{125}I and 3 μmole H_2O_2 added over a 1 h period in 0.2 μmole aliquots. Butylated hydroxytoluene (4 μgm/ml) was added as antioxidant and the reaction was terminated with 1 μl 14.3 M β-mercaptoethanol.

Band No	1	2	3	4	Comments
1					
2			+		
3	+	+	+		
4		+	+		
5					
6					
7			} +		
8					
9		+			
10					
11			+		
12			+		
13	+	+	+		This region is very
14	+	+	+		DβH poorly labelled compared
15	+	+	+		to its staining intensity.
16	+	++	+	+	
17	++	+	+	+	
18	+	+	+	+	
19	+++	+ ++	++	+	
20	++	+	+	+	
21	++nr	++	+	+	
22	++	+	+	+	
23	++	+	+	+	
24	+++	+++	++++	++	
25	?	++++	+++	++	
26	++++	+++++	+++++	++	
27	++++?	+++	+++?	++	
28	+++	+++++	++	++	ATPase subunit
29	++++	++	+	++?	ATPase subunit
30	+++	+++	++	++	
31	++++	+++	+ ++	++	
32	+++	+++	+	+	
33	++++	+++	+	+	
34	+++	+++	+	+	
35	++ +++	++ ++	+	+	
36	++		+	+	
37	++	++	+		
38	+	++	++		
39	+	+	++	+	
40	++	+	+?		
41	} +	} +	} +		
42					
43	+	+	+		
44			+		ATPase subunit
45		+			
46	+	++		+	
47			+ +		
48		+			
49	+++++ +	++++	+	+++++	} Very heavily labelled minor
50		++	++	nr	} staining band
51	+ +		+	nr	} CHROMOMEMBRIN B

'HUMP' REGION OF BACKGROUND

Table 6.1 Lactoperoxidase Catalyzed Radioiodination of the Membrane Polypeptides of Intact Chromaffin Granules I

For details see text

incorporation of ^{125}I into the membranes a general pattern of labelling of granule membrane constituents is usually observed. The radioactive profile can be divided into three regions roughly coresponding to the top, middle and bottom sections of the gel. In the first region, occupied by CBB stained bands 1 to 15, many small radioactive peaks are observed. These peaks are all similar in size and there are more of these peaks than there are CBB stained bands in this region. It is suggested that this may represent background 'noise' i.e. variations in the background radioactivity of the gel presumably caused by the 'slicing and counting' method of estimating radioactivity. (As in the CBB staining profile of the gel there is a very high background to the radioactivity profile throughout the gel. A normal background count is 40 - 50 cpm which is 4 - 5 times lower than the background in the gel). The most heavily stained bands (13, 14 and 15) which comprise the subunits of DβH occur in this region of the gel. Thus DβH does not appear to be labelled when chromaffin granules are iodinated by lactoperoxidase.

The middle region of the gel is dominated by an extremely broad radioactive band which in the case of the experiment depicted in Fig. 6.1 stretches from CBB stained bands 12 to 44. Superimposed upon this are many sharper peaks giving the impression that this band represents an elevated background count. The band, however at its centre (CBB stained bands 29, 30) contains twice as much radioactivity as the background in the rest of the gel. It shows a Gaussian profile and is clearly not due to any iodinated CBB stained band or series of bands. The significance of this 'hump' in the radioactive profile background is unknown. (However, we can note at this point that the CBB staining profile of gels of reduced granule membranes also shows broad

areas of elevated background staining). The sharper radioactive peaks superimposed on the hump probably represent iodinated CBB stained bands in this region of the gel (see later). The hump was not as pronounced in experiments 3 and 4 of Table 6.1. although it was clearly present. Lower amounts of ^{125}I were incorporated into the granule membrane in these two experiments.

The largest radioactive peak in the radioactive profile of granule membranes prepared from radioiodinated granules occurs in the bottom region of the gel (except in experiment 3 of Table 6.1). Like the majority of the CBB stained bands this peak is sharply resolved and co-migrates with band 49 of the reduced granule membranes. CBB stained band 49 is a minor staining component and, together with bands 50 and 51, is thought to comprise the low molecular weight major CBB staining component of SDS gels of granule membranes run by Winkler and termed 'chromomembrin B' by him (see chapter 3). Bands 50 and 51 are also clearly labelled, although to a lesser extent than band 49. Another relatively minor staining component in this region of the gel, band 46, is also consistently iodinated in experiments 1 to 4 of Table 6.1. In contrast CBB stained bands 44, 45, 47 and 48 of the reduced membranes are usually only associated with background levels of radioactivity and thus are not iodinated when intact granules are labelled with lactoperoxidase.

In Table 6.2 the results of experiments in which granule membranes have been iodinated by lactoperoxidase in granules have been analyzed in a different way to that used in Table 6.1. CBB stained bands of the reduced membranes have been assigned either a plus or a minus according to whether they co-migrate with peaks or troughs respectively in the radioactive profile of the membranes. The actual amount of radioactivity

Band No	1	2	3	4	Comments
1	-	-	s	nr	
2			-	nr	
3	+	+	+	nr	
4	+	+ -	+	nr	
5	+	+	-	nr	
6	-	-	-	nr	
7	+	+ nr	+	nr	
8		nr		nr	
9	+	+	nr	nr	
10	-	-	nr	nr	
11	+	+	-	nr	
12		-	+	nr	
13	+?	+	+	?	DβH
14	+	+	+	?	
15	+ -	s	-	?	
16	-	+	-	+	
17	+	-	s?		
18	-	-	s?		
19	+	- +	+		
20	nr	-	-	-	
21	nr	+	-	-	
22	+	-	-	s?	
23	-	-	-	?	
24	+	s	+++	+	
25	?	?	?	s?	
26	++	++	+++	+	
27	+	-	?	-	
28	-	++	?	+	ATPase subunit
29	+	-	-	-	ATPase subunit
30	-	++	+	+	
31	+		-	+	
32	-	-	++	-	
33	++	+	+	+	
34	+	s	-	-	
35	-	-	+	-	
36	+ -	+ s?	s?	+	
37	+	s?	s?	?	
38	-	-	+	-	
39	-	+	+	+	
40	+	+	s?	+	
41	-	+	-	+	
42					
43	+	+	+	+	
44	-	-	s?	-	
45	+?	s	s?	-	
46	++	++	-	+	
47	-	-	+ ?	-	
48	-	+	-	-	
49	+++++	+++	?	++++	CHROMOMEMBRIN B
50	+ -	- +	+	nr	
51	+ +	- +	+	nr	

Table 6.2 Lactoperoxidase Catalyzed Radioiodination of the Membrane Polypeptides of Intact Chromaffin Granules II

For details see text

nr - not resolved ; s - shoulder

associated with each CBB stained band is ignored. This type of analysis effectively allows one to treat the 'hump'in the radioactive profiles of the membranes as shown in Fig. 6.1 as background radioactivity. Because the 'hump' is ignored we can interpret the significance of the sharper peaks superimposed on the hump in relation to the CBB bands. One can also decide whether any of the smaller peaks present in the top region of the gel of membranes are due to the presence of any of the CBB stained bands in this region.

Thus when chromaffin granules are labelled CBB stained bands 3,4,5,7-8, 9,13 and 14 of reduced granule membranes are associated with small radioactive peaks. In contrast CBB stained bands 1,2,6,10 and 15 do not appear to be labelled even to a minor extent. The labelling pattern of CBB stained bands 11 and 12 is uncertain. There is usually a small radioactive peak in this area but whether it is associated with band 11 or 12 or both is uncertain. When deciding whether a band is iodinated one must bear in mind that, because there are so many small peaks in this region the chances of a CBB stained band co-migrating with a radioactive peak are relatively high.

In the 'hump' region of the gel the sharper radioactive peaks are consistently associated with CBB stained bands 19, 24, 26, 28, 30,31, 33, 39, 40 and 43. In addition a radioactive peak is consistently located between CBB stained bands 35 and 36. Whether this peak is due to mis-alignment and is due to the presence of band 35 or 36 is uncertain. CBB stained bands 18, 20, 21, 22, 23, 29, 32, 34, 35 and 38 do not usually co-migrate with radioactive peaks. Analysis of the labelling properties of other CBB stained bands in this region is uncertain. A radioactive peak is usually associated with either bands 16 or 17 and it is impossible to decide to which band this radioactive peak is due.

Bands 25 and 27 occur close to CBB stained bands 24 and 26 respectively; these are both iodinated and this makes it difficult to decide whether bands 25 and 27 themselves are labelled with ^{125}I. Bands 36 and 37 are either associated with a clearly defined radioactive peak or the shoulder of a peak, while bands 41 and 42 which co-migrate are associated with radioactive peaks in experiments 2 and 4 and with troughs in experiments 1 and 3.

In agreement with Table 6.1, in the bottom region of the gel CBB bands 46, 49, 50 and 51 are iodinated (or at least are associated with sharply resolved radioactive peaks), although the alignment of radioactive peaks with CBB stained bands 50 and 51 is occassionally askew. CBB stained bands 44, 47 and 48 are unlabelled whilst band 45 may be iodinated to a minor extent.

Lipid and the 'tracker dye' regions of gels of granule membranes (reduced) are also iodinated when granules are labelled. Radioactivity co-migrating with the tracker dye is probably mainly due to free iodide although iodinated proteins of molecular weight 18,000 daltons or less (see chapter 2,3) would also be expected to migrate with the tracker dye. The proportion of radioactivity in each profile associated with lipid is indicated in Table 6.10 and must be viewed as a minimum percentage since lipids are probably lost from the gel during staining and destaining.

6.3.1.2 Autoradiography

Radioactivity in polyacrylamide gels has also been localized by autoradiography of the dried down gel. The gel shown in Fig. 6.2.1 containing a sample of granule membrane prepared from iodinated granules (lane 4) and separated by SDS polyacrylamide gel electrophoresis under reducing conditions, produced the autoradiogram pictured in Fig. 6.2.2 (lane 4). The labelled membranes were the same as those (used to obtain

	Protein	Dye Front	Lipid
Granules:			
Expt 1	85.9%	6.4%	7.7%
Expt 2	77.9%	6.4%	15.7%
Expt 3	n.d.	n.d.	n.d.
Expt 4	73.4%	5.4%	21.2%
Granule Membranes:			
Expt 1	80.5%	7.5%	12%
Expt 2	72.9%	8.2%	18.9%
Expt 3	68.5%	7.9%	23.6%
Expt 4	69.7%	7.3%	23%
Expt 5	66.2%	7.9%	25.9%

Table 6.10 Lipid Labelling During Lactoperoxidase Catalyzed Radioiodination of Granules and Granule Membranes

The three figures given in each experiment for protein, dye front and lipid labelling represent the percentage of total radioactivity in each gel of labelled memberanes migrating behind the dye front, at the dye front and ahead of the dye front respectively. Experiments referred to are those of Tables 6.1 and 6.2 and 6.6 and 6.7.

Fig. 6.2 Localization of Radioactive Bands by Autoradiography

SDS polyacrylamide gel electrophoresis was performed in 8% (w/v) acrylamide gels under reducing conditions. The gel contains membranes iodinated with lactoperoxidase immediately after preparation (lane 2), membranes iodinated with lactoperoxidase after storage at -20°C (lane 1), membranes prepared from resealed granule membrane ghosts iodinated with lactoperoxidase (lane 3), membranes prepared from intact granules iodinated with lactoperoxidase (lane 4), membranes prepared from intact granules iodinated with lacto - peroxidase (lane 5), molecular weight markers (lane 6), and untreated chromaffin granule membranes. Protein was detected with coomassie brilliant blue after which the gel was dried down and autoradiographed. Iodinated membranes separated in lanes 2, 1, 3 and 4 were the same as those used in Fig. 6.6, 6.7, 6.10 and 6.1 respectively. The membranes separated in lane 5 were prepared from granules iodinated with lactoperoxidase as described in Fig. 6.1 except that 0.08 units of lactoperoxidase were used.

Fig 6.2.1.

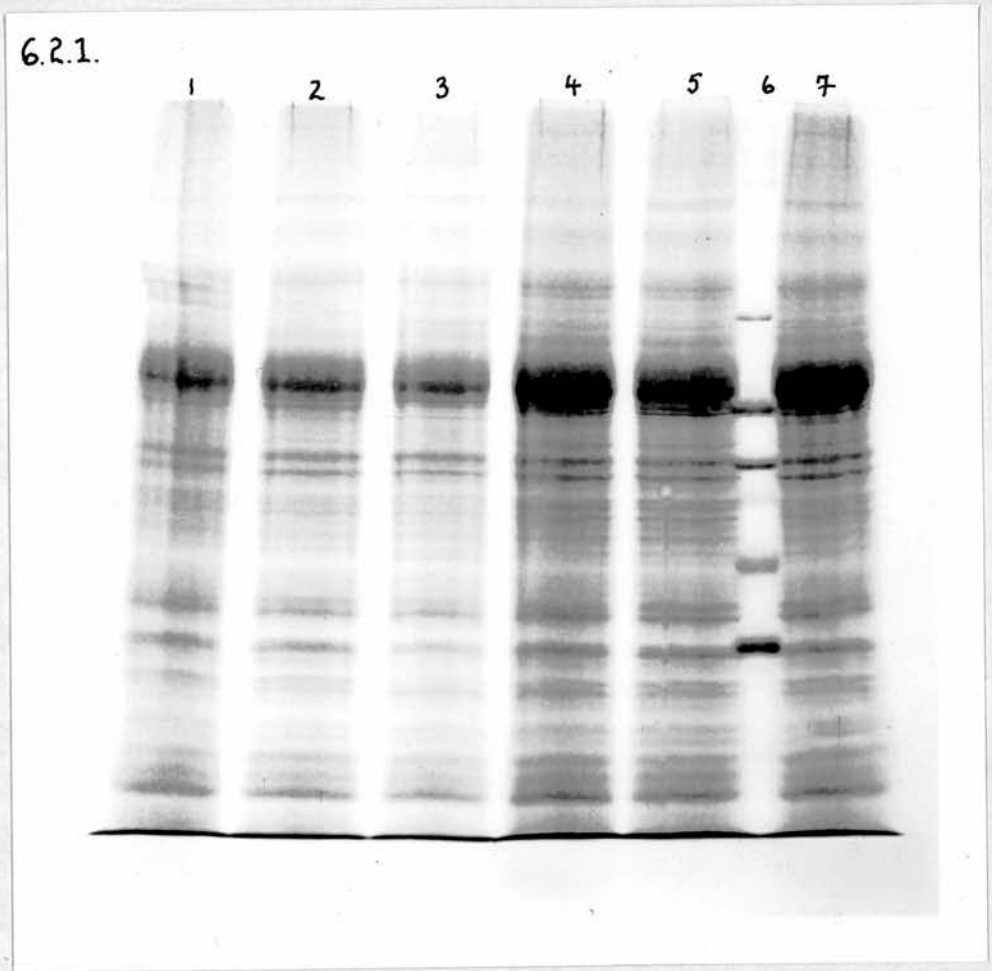
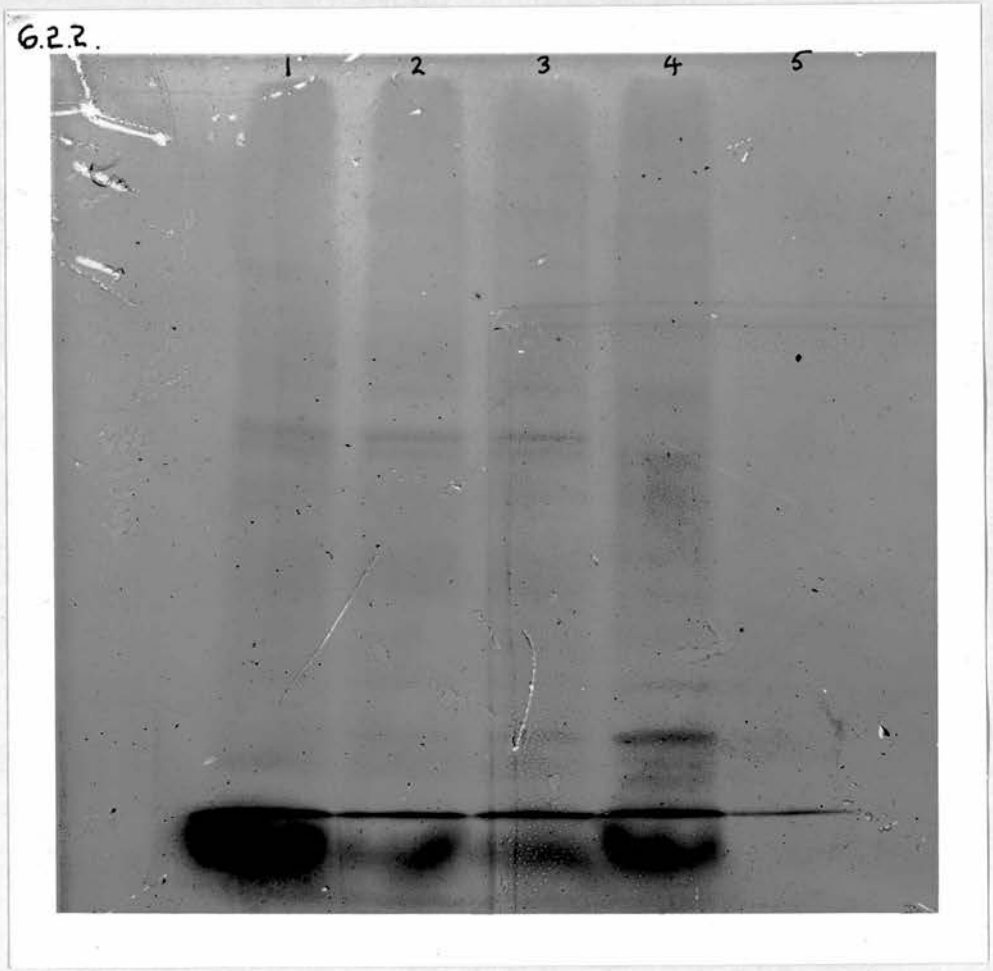


Fig 6.2.2.



the profile)shown in Fig. 6.1 and analyzed in experiment 1 of Tables 6.1 and 6.2. The staining profile and autoradiogram have been compared in Table 6.3, lane 4.

Despite the underexposure and poor quality of the X-ray film, one can clearly see several features observed in the radioactive profile of granule membranes, labelled in the granule, produced by the 'slicing and counting' method. One can see the high background which highlights the sample lane from the surrounding gel even where no radioactive bands are observed. The hump region of the radioactive profile, the prominently labelled low molecular weight component (band 49) and the lack of radioactive bands in the top third of the gel are also easily observed. The most heavily 'labelled' regions in the 4th lane of the autoradiogram are occupied by the tracker dye and lipid. (Note that lipid labelling is considerably greater here than is indicated in Table 6.10 even though the gels are of the same iodinated membrane sample. This is probably because lipid is not 'fixed' in the gel and is eluted, to varying degrees, from the gel during staining and destaining).

In the first third of lane 4 of the autoradiogram very faint bands can be seen which correspond with CBB stained bands 3, 4 to 6, 7 and 8, and 14. Bands 4, 5 and 6 are represented by a single broad band in the autoradiogram, as are bands 7 and 8. There may be radioactive bands in the region occuppied by CBB stained bands 11, 12 and 13 but this region in the autoradiogram is obscured by a mark.

A few individual bands are seen in the middle section of the autoradiogram containing the 'hump' in the background radioactivity level and these correspond to CBB stained bands 24, 26 and 27. In addition there is an extremely faint radioactive band migrating just behind CBB stained band 24 and this could be due to labelling of band 22 or 23 of the reduced granule membrane staining profile. CBB stained band

	F.T. GMs (Lane 1 Fig 6.2)	Fresh GMs (Lane 2 Fig. 6.2)	Ghosts (Lane 3 Fig 6.2)	GMs from ^{125}I Gs (Lane 4 Fig 6.2)
1				
2	+ v.faint	+ v.faint	+ v. faint	
3	+	+ v faint	+ v. faint	+ v. faint
4				
5	+ faint but more intense than lanes2-4	+ v.faint	+ v. faint	+ v. faint
6				
7				
8				?
9				
10				
11				
12	+	+ v. faint	+ v. faint	↑? ↓
13				
14	+	+ broad, poss-	+	↑?
15		ibly includes		
16				
17	+	+ or 18	+	
18				+ v broad
19				
20				
21				
22				
23				
24	++++	++++ v. strong	++++	+ faint
25				
26	+++	++++ v. strong	+++	+
27				+
28				
29				↑?
30	+	+ broad,weak	+	+ broad band
31				↓?
32	+			
33				
34				
35	+extends be-	?extends be-	extends be-	↑?
36	tween 35-36?	tween 35-36?	tween 35-36?	+ broad
37				
38	+	+ broad,diffuse	+	
39		band - no in-		
40		dividual bands seen superimposed		+ broad
41				
42	+? v. faint	+? v. faint	+? v. faint	+? v. faint
43	+	+	+	+ broad
44				
45				
46	+	+	+	++ moderate
47				
48				
49	+	+	++	++++ v.heavy
50	not clearly	+	+	++ moderate
51	+ resolved	+	+	++ moderate

HIGH BACKGROUND

Table 6.3 Localization of Radioactivity by Autoradiography

For details - see text and Fig. 6.2

26 is the most heavily labelled band in the hump region while band 24 is only labelled to a minor extent. Although other sharply resolved bands are not seen in this region of the autoradiogram there are several other broader labelled components. Thus CBB staind bands 16-19 are associated with a broad moderately intense band of the autogradiogram as are bands 29-31 although this radioactive band is more intense. Another broad radioactive band superimposed upon the 'hump' occurs in the CBB stained bands 35-36 region whilst CBB stained bands 39-40 are also associated with a radioactive band. In between these two radioactive bands there is a clear depression in the profile indicating a lack of labelling of the CBB stained bands in this region (bands 37 and 38) or, at least a relatively smaller amount of labelling compared to bands 35, 36, 39 and 40. Either band 41 or 42 or both are labelled to a very minor extent. Bands 41 and 42 run as a single band or very close doublet and are relatively well stained by CBB. In contrast, band 43 a minor CBB staining component is slightly more heavily labelled.

At the bottom end of the autoradiogram, i.e. the region immediately behind the tracker dye and lipid areas, 4 radioactive bands are indicated corresponding to CBB stained bands 46, 49, 50 and 51. All are moderately intense except for band 49 which is very heavily labelled. In addition the background between bands 49, 50, 51 and the tracker dye is quite high. In contrast to Fig. 6.1 bands 50 and 51 co-migrate exactly with the radioactive bands seen in their region of the gel. CBB stained bands 44, 45, 47 and 48 are not labelled.

6.3.1.3 Non-reducing Gels

The radioactive distribution in gels of granule membranes prepared from iodinated granules is similar under non-reducing conditions of electrophoresis to that under reducing conditions (Fig. 6.3) even though

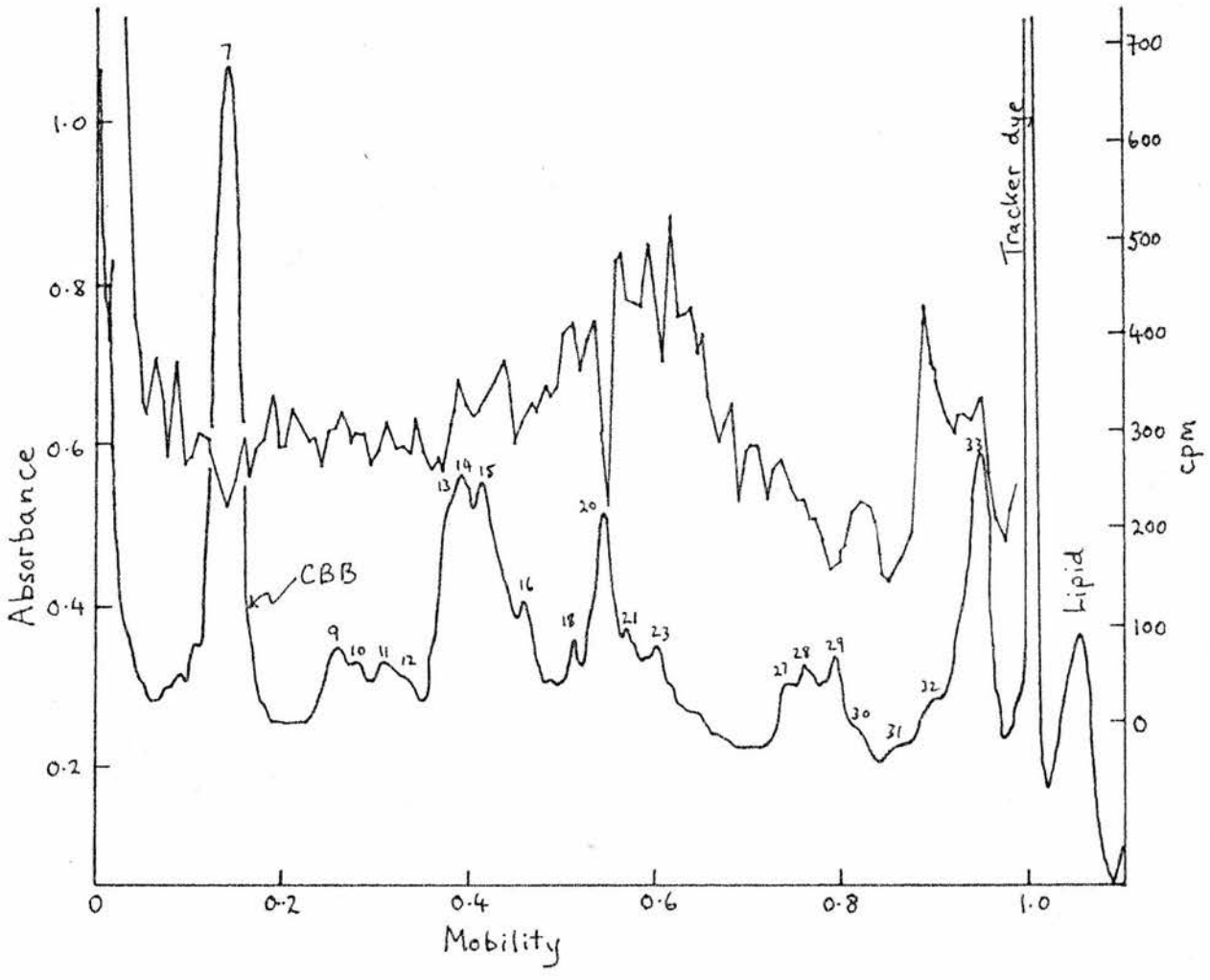


Fig.6.3 Distribution of Radioactivity in Non-Reducing Gels of Membranes of Lactoperoxidase Iodinated Chromaffin Granules

The membranes of lactoperoxidase iodinated chromaffin granules (see Fig. 6.1 for further details) were separated by SDS polyacrylamide gel electrophoresis under non-reducing conditions. Protein was determined by densitometric scanning of the CBB stained gel at 570 nm and radioactivity by slicing and gamma spectrometry.

the CBB staining profiles are different. Thus few radioactive bands are seen in the first third of the profile, followed by a hump with sharper peaks superimposed and finally a region containing sharply resolved radioactive peaks. Much radioactivity remains at the top of the gel and is presumably due to iodinated CBB stainable material which also fails to enter the gel.

The use of 'non-reducing gels' is useful in confirming whether dopamine-β-hydroxylase is labelled or not. Label associated with the DβH region in reducing gels of granule membranes should also co-migrate with the new position DβH occupies in non-reducing gels. In non-reduced gels of granule membranes prepared from granules iodinated by lactoperoxidase no radioactivity is associated with CBB stained band 7, the dimeric form of dopamine-β-hydroxylase, and, therefore, radioactivity associated with the DβH region in reduced gels is probably due to other iodinated components migrating with DβH under reducing conditions. Thus we can conclude that the membrane form of DβH is not labelled when intact granules are iodinated by the lactoperoxidase procedure.

Winkler's chromomembrin B has been identified as CBB stained bands 49, 50 and 51 of the reduced staining profile of the membranes. The mobility of chromomembrin B is reported to be unaffected by the presence or absence of reducing agent during electrophoresis and I have identified CBB stained bands 31, 32 and 33 of the non-reduced profile as corresponding to bands 49, 50 and 51 respectively of the reduced profile. The non-reduced staining bands 31, 32 and 33 should, then have the same labelling profile as the reduced bands 49, 50 and 51 when the same iodinated membranes are electrophoresized under these conditions. Non-reduced bands 32 and 33 are labelled to the same extent as reduced bands 50 and 51 (in relation to the rest of the radioactive profile which as

noted earlier is similar under reducing and non-reducing conditions). However, non-reduced band 31 is relatively less labelled than reduced band 49 of the membranes to the extent of about 50%. Therefore reduced band 49 may comprise two polypeptides both of which are capable of being iodinated when intact granules are labelled. Under non-reducing conditions these bands migrate independently, only one of them migrating with a similar mobility to that under reducing conditions(non-reduced band 31). No new radioactive band is observed in the non-reduced radioactive profile, nor is any radioactive band increased in intensity. Therefore the other polypeptide of reduced CBB stained band 49 either remains at the top of non-reduced gels or migrates with the tracker dye, bromophenol blue.

The radioactive profile and staining profile of non-reducing gels of granule membranes iodinated in the granule state are compared in Table 6.4. As for Table 6.1, non-reduced CBB bands have been awarded between one and five pluses according to the amount of radioactivity associated with them. Because of the uncertain relationship between CBB stained bands in non-reduced gels and the proteins and/or polyp peptides of the chromaffin granule membrane (see chapter 3) the results of iodination experiments with lactoperoxidase have been expressed in terms of labelling of reduced CBB staining bands of the granule membranes. However, the use of non-reducing gels to separate granule membranes does provide confirmatory information about the labelling of dopamine-β-hydroxylase and the polypeptides comprising Winkler's chromomembrin B. As expected both the tracker dye and lipid regions are labelled when granule membranes prepared from iodinated granules are separated under non-reducing conditions of electrophoresis.

The iodinated granule membrane sample separated under non-reducing

Non-Reduced Band No	Reduced Band No	Granules	Granule Membranes	Comments
1		+++++++	+++++++++	
2		+++++	+++++	
3		++	++	
4			+	
5		++		
6		+	+	
7		+	+++++	DβH
8			+	
9	(6)			
10	(7,8)			
11	(12)			
12	(14?)			
13	(15?)	++	++	
14	(16,17?)	++	+++	
15	(22?)	++	+	
16	(24,25?)	++		
17	(26,27?)	++		
18		+++	+	
19		+++	+	
20	(28,29)	++	++	
21	(30,31)	++++	+	
22	(32-35)	++++		
23		+++		
24		++++		
25	(36)	+++		
26	(37)	++		
27	(41,42)	++		
28	(43)	+		
29	(44,45)			
30	(46)	+		
31	(49)	++++	+	CHROMOMEMBRIN B
32	(50)	++	+	
33	(51)	++	++	
34			+	

Table 6.4 Lactoperoxidase Catalyzed Radioiodination of Granule Membranes - Analysis of Non-Reduced Polypeptides I

For details - see text and Fig. 6.3 and 6.8

Non-Reduced Band No	Reduced Band No	Granules	Granule Membranes	Comments
1		+++++++	++++++++++	
2		?	s	
3		-	s	
4		-	+	
5		+		
6		+	s?	
7		-	++++	DβH
8		+	s?	
9	(6)	+	+	
10	(7,8)	+	-	
11	(12)	+	-	
12		+	+	
13	(14?)	-	+	
14	(15?)	+	+	
15	(16,17?)	-	+	
16	(22?)	+ -	s?	
17	(24,25?)	-	-	
18	(26,27?)	+	+	
19		-	+	
20	(28,29)	+ -	++	
21	(30,31)	+	+	
22		+	?	
23	(32-35)	-	?	
24		+	?	
25	(36)	?	?	
26	(37)	-	?	
27	(42,41)	+	+	
28	(43)	?	-	
29	(44,45)	-	-	
30	(46)	+	+	
31	(49)	++	+	CHROMOMEMBRIN B
32	(50)	+	+	
33	(51)	+	++	
34		+	+	

Table 6.5 Lactoperoxidase Catalyzed Radioiodination of Granule Membranes - Analysis of Non-Reduced Polypeptides II

For details - see text and Fig. 6.3 and 6.8

conditions here was the same membrane sample as that used to produce the reduced radioactive profile of iodinated granule membranes shown in Fig. 6.1. The non-reduced staining profile of granule membranes was similar to that previously described (see chapter 3).

6.3.1.4 Incorporation of Radioactivity into Proteins of the Granule Lysate.

Granule lysate was also prepared from granules iodinated by the lactoperoxidase procedure and subjected to SDS polyacrylamide gel electrophoresis under reducing conditions. The radioactive profile of a sample of this lysate, determined by the 'slicing and counting' method is shown in Fig. 6.4 as is the CBB staining profile of the lysate. Very little radioactivity has been covalently bound to the proteins of the lysate. The general background radioactivity to the gel of the lysate proteins is the same as that of a 'natural' radioactive background (40 - 50 cpm) unlike the background radioactivity of gels of granule membranes prepared from iodinated granules. Peaks in the radioactive profile of the lysate, corresponding to chromogranin A and the second major CBB staining band are less than one and a half times the background count. A significant amount of iodine is associated with the tracker dye and this is probably due to free iodide.

6.3.1.5 ATPase

The subunits of the Mg^{++} ATPase of chromaffin granule membranes can be partially purified by extracting the membranes with dichloromethane. These subunits have been identified as CBB stained bands 28, 29 and 44 of reduced granule membranes. Other bands present in this partially purified preparation include bands 3,5,6,8,9,12,13,14,15, 16,17,22,24,38,40,42,46,47 and 48 (see Fig.6.5). When granule membranes

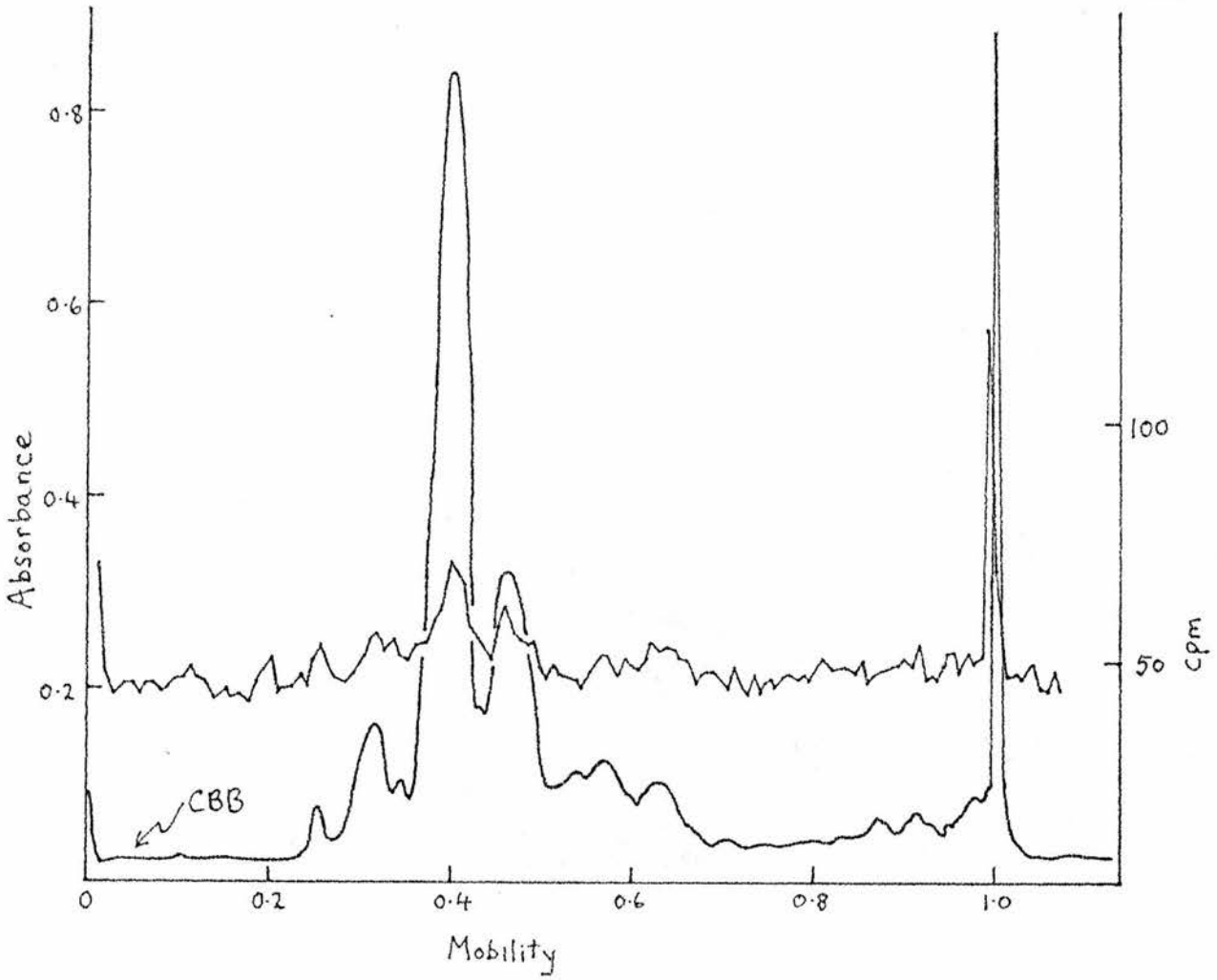


Fig. 6.4 Distribution of Radioactivity in Gels of Granule Lysate of Lactoperoxidase Iodinated Granules

Granule lysate was prepared in the normal manner (see section 2.1.2) from granules iodinated with lactoperoxidase as described in Fig. 6.1. SDS polyacrylamide gel electrophoresis was performed under reducing conditions in 8% (w/v) acrylamide gels, protein was detected by densitometric scanning of the CBB stained gel and radioactivity determined by gamma spectrometry of uniform slices of the gel (c 1 mm thick).

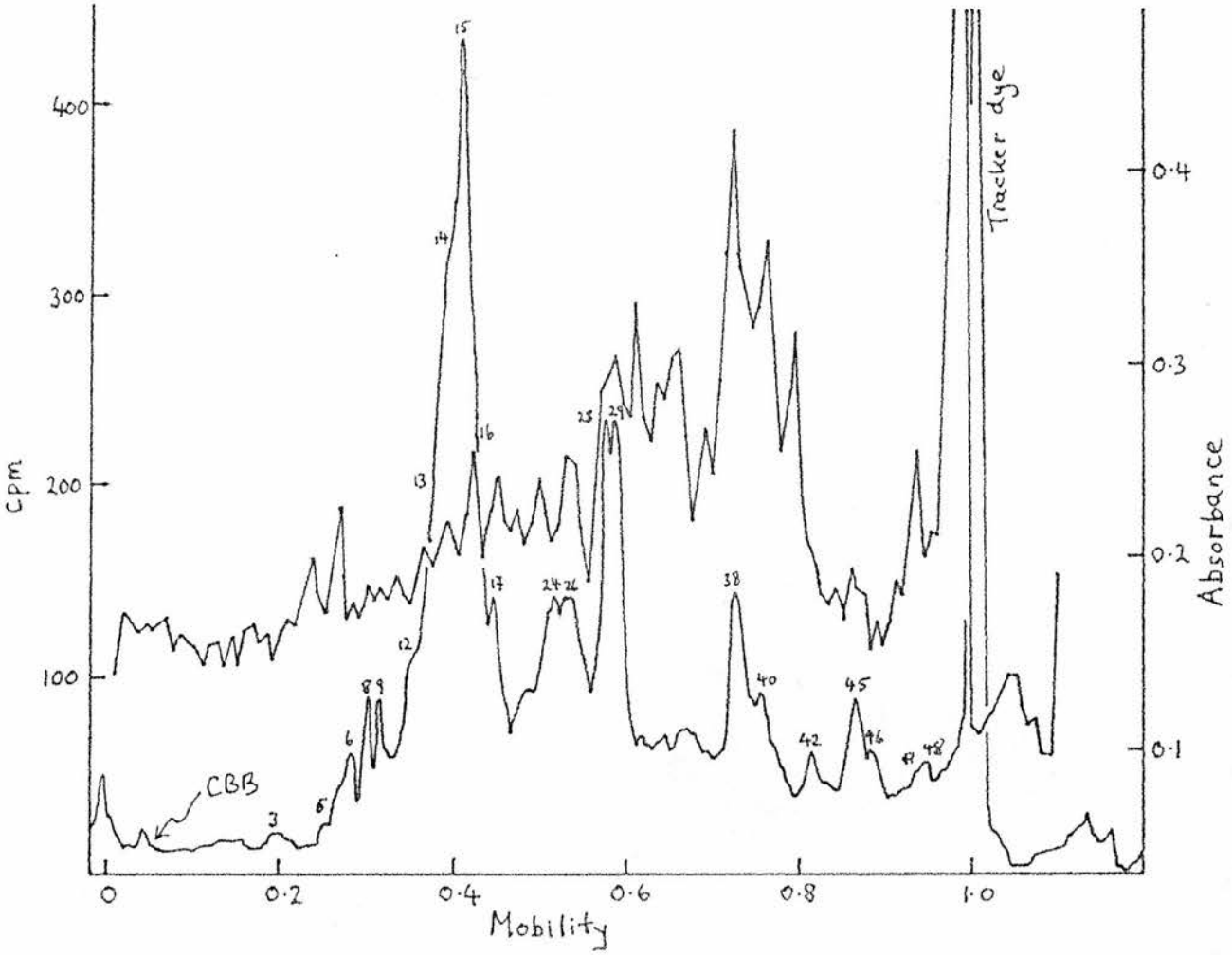


Fig. 6.5 Lactoperoxidase Catalyzed Iodination of the Membrane Bound Mg^{++} ATPase in Intact Chromaffin Granules

Membranes prepared from intact granules iodinated using lactoperoxidase (see Fig. 6.1) were extracted with dichloromethane according to Apps and Glover (1978). The residue was subjected to SDS polyacrylamide gel electrophoresis in 8% (w/v) acrylamide gels under reducing conditions. The gel was stained for protein with CBB and then scanned at 570 nm after which radioactivity in the gel was localized by sectioning the gel into c 1 mm thick slices and counting each slice directly in a gamma spectrometer. The ATPase subunits are CBB stained bands 28, 29, and 44.

prepared from lactoperoxidase iodinated granules are extracted with dichloromethane and the extracted membranes subjected to SDS polyacrylamide gel electrophoresis much radioactivity remains with CBB bands 28 and 29 but band 44 does not appear to be labelled. CBB bands 38, 40 and 42 are the most heavily iodinated components in dichloromethane extracted membranes.

6.3.2 The Labelling of Purified Chromaffin Granule Membranes by the LPO/H_2O_2/^{125}I Procedure

6.3.2.1 Iodination of freshly prepared granule membranes

Purified chromaffin granule membranes not subjected to freezing and thawing have been iodinated by the lactoperoxidase procedure within a day of their preparation. Fig. 6.6 shows the radioactive distribution in a reducing gel of the labelled membranes, produced by the 'slicing and counting' method and the CBB staining profile of the gel. The staining and radioactive profiles are compared in column one of Tables 6.6 and 6.7. In Table 6.6 between one and five pluses are assigned to each CBB stained band according to the amount of radioactivity associated with the band and in Table 6.7 pluses or minuses are assigned to each band according to whether they co-migrate with peaks or troughs respectively in the radioactive profile.

The radioactive and CBB staining profiles of gels of iodinated granule membranes are different. The major staining region of the gel, CBB stained bands 13, 14 and 15 (DβH) is only moderately labelled whilst bands 24 and 26, two sharply resolved moderately stained components migrating just ahead of DβH, are very heavily iodinated. There is a high radioactive background to gels of iodinated granule membranes (approximately 4 to 5 times the 'natural' background). The radioactive background is more even although a relatively small broad hump

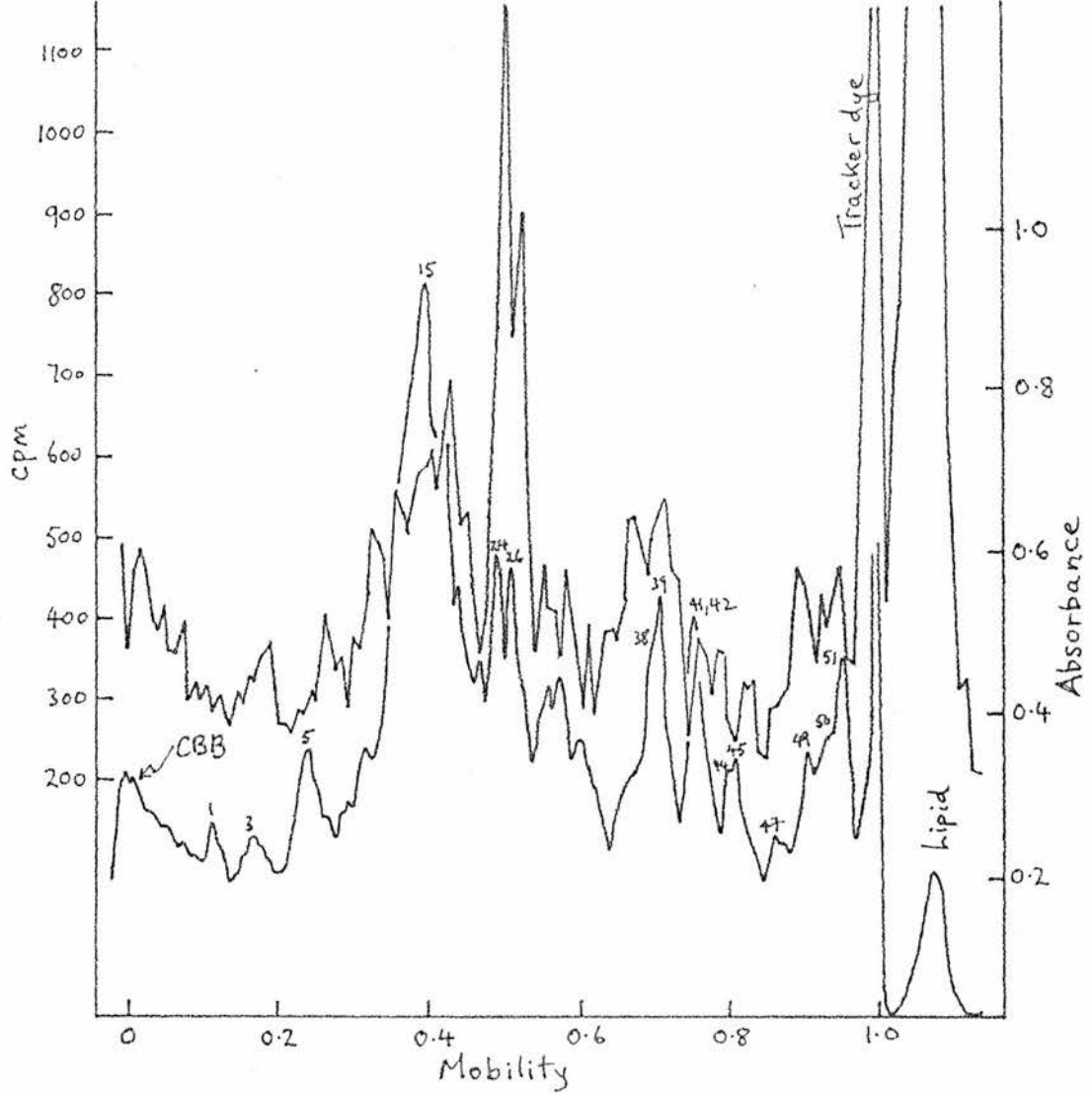


Fig. 6.6 Distribution of Radioactivity in Reducing Gels of Granule Membranes Iodinated with Lactoperoxidase Immediately After Purification

Freshly prepared granule membranes (2 ml, 1 mg/ml protein in 10 mM hepes, pH 7), not subjected to freezing and thawing, were iodinated at room temperature with 200 μCi Na ^{125}I, 0.08 units lactoperoxidase and 0.75 μmole H_2O_2 added in 15 0.05 μmole aliquots at 4 min intervals. The antioxidant, butylated hydroxytoluene was present at 2 μgm/ml and the reaction was terminated with 1 μl 14.3 M β-mercaptoethanol. The washed radioiodinated membranes were separated by SDS polyacrylamide gel electrophoresis under reducing conditions in 8% (w/v) acrylamide gels. Protein was detected by staining with CBB followed by densitometric scanning at 570 nm. Radioactivity was localised by slicing and gamma spectrometry.

Band No.	1	2	3	4	5	Comments
1			nr			
2		++++	nr			
3	+	+	+	+		
4		++	+			
5	+		+			
6	+	+	+	+		
7			+			
8			+			
9			+			
10			+			
11	++	+	+		+	
12			+	+	+	
13	++	+++	++	++	++	DβH
14	++	++	++	+	+++	
15	++	++	++	++	+++	
16	+++	+++	++	++	+++++	
17	++	++	+++	++		
18	++	+++	++	+	+	
19	+?	+	+++	+	+	
20	+?	nr	++	nr	+	
21	?	nr	++	nr	+	
22	?	++	+++++	nr	+	
23	?	+	++	nr	+	
24	+++++	++++	+++++	+++++	+++++	
25	??	?	++	?	+	
26	++++	+++++	+++++	++++	++++	
27	??	?	++	?	+	
28	+	?	++	+	+	ATPase subunits
29	+	?	++	+	+	
30		?	++		+	
31	+	?	++	+	+	
32		+	++	+	+	
33	+	+	++		+	
34		+	++	+	+	
35	+	+				
36	+	++?	++	+?	++	
37	++	++	+	+++	+++	
38	+	++	+++	++	++++	
39	++	++	++	+++	+++	
40	+	+	+	++	++	
41	+	+		+	+	
42						
43	+?	+	+	+	+	
44						ATPase subunit
45						
46	+	+	+	+	+	
47	+	+				
48	+	+				
49	++	+	++	++	++++	CHROMOMEMBRIN B
50	++	++	+++	++	nr	
51	++	++++	nr	+++	nr	

Table 6.6 Lactoperoxidase Catalyzed Radioiodination of Purified Granule Membranes I

Band No	1	2	3	4	Comments
1	-	-	nr	+	
2	-	++++	nr	+	
3	+	+	+	+	
4	-	++	-	+	
5	+	-	+	-	
6	+	+	nr	+	
7	-	-	nr	+	
8	-	+	nr		
9	+?	-	nr	-	
10		s?	nr	-	
11	+	s?	nr	+	
12		+	nr		
13	+	+	+	+	DβH
14	s?	-	+	-	
15	+	-	+	-	
16	+	+	-	+	
17	-	-	+		
18	+	+	+	-	
19	-	-	-	-	
20	-	nr	nr	nr	
21	-	nr	nr	nr	
22	-	+	+++++?	nr	
23	-	-	?	nr	
24	+++++	++++	+++++	+++++	
25	-	-	-	-	
26	++++	+++++	+++++	++++	
27	-	-?	-	-	
28	+	-?	-	+	ATPase subunits
29	s+	-?	-	+	
30	-	-?	+	-	
31	+	?	-	+	
32	-	-	s?	+	
33	+	+	-	-	
34	-	-	+	+	
35	-	+	-	-	
36	+	+	+?	+	
37	+	+	-	+	
38	-	+	+	-	
39	+	+	++	+	
40	s?	-	-	+	
41	+	s?	-	+	
42					
43	+	+	+	+	
44	-	-	-	+	ATPase subunit
45	-	-	-	-	
46	+	+	+	+	
47	s?	+	-	+	
48	s?	s?	-	-	
49	+	s?	+	+	CHROMOMEMBRIN B
50	+	+	++	-	
51	+	++++		+	

Table 6.7 Lactoperoxidase Catalyzed Radioiodination of Purified Granule Membranes II

nr - not resolved ; s - shoulder

can be seen in a similar position to that in membranes prepared from iodinated granules. The peaks superimposed on this hump are much larger and more clearly defined when purified membranes are labelled. CBB stained band 49, which is very heavily labelled in granules, is poorly labelled by comparison when membranes are iodinated. At the bottom of the gel the tracker dye and lipid regions are associated with large amounts of radioactivity.

Thus from Tables 6.6 and 6.7 we may conclude the following about the labelling characteristics of 'fresh' L P-iodinated granule membranes. CBB stained bands 1 and 2 are not labelled whereas band 3 is associated with a small, broad radioactive peak similar in shape to the densitometric scan of this CBB stained band. Of bands 4 to 8 only bands 5 and 6 are labelled even to a minor extent. A broad radioactive peak with a sharper superimposed peak is associated with the region occupied by CBB stained bands 9 to 21. There is a small peak on the edge of this which could be due to CBB stained band 9 or 10. A broader peak of slightly larger height is associated with CBB stained bands 11 and 12. Other CBB stained bands associated with peaks superimposed on the broad peak are bands 13, 15, 16 and 18. Band 16 is particularly well labelled in this region whilst band 14 is associated with a shoulder in the radioactive profile associated with the radioactive peak presumed to be due to CBB stained band 15. It is uncertain whether CBB stained bands 22, 23, 25 and 27 are labelled or not. They occur extremely close to the heavily iodinated bands 24 and 26 in the gel which would obscure minor amounts of radioactivity associated with these bands especially band 15. Further down the gel CBB stained bands 28,29, 31 and 33 are iodinated to a minor extent whereas bands 30, 32, 34 and 35 are not. Another broad radioactive band with smaller, sharper

radioactive peaks superimposed is present in the region of the gel stretching from CBB stained bands 35 to 40. The sharper peaks occur between bands 35 and 36 and at bands 37 and 39. A shoulder at the edge of the broad peak corresponds to band 40. The shape of this broad radioactive peak is entirely different to the densitometric scan of this stained region. In particular the minor staining bands 37 and 40 are more heavily iodinated. In the last region of the gel, just behind the tracker dye, CBB stained bands 49 and 51 are well labelled, bands 41-42, 43 and 46 iodinated to a minor extent and bands 47 and 48 are associated with a shoulder on the radioactive peak associated with CBB stained band 49. Bands 44 and 45 are not labelled and are associated with troughs in the radioactive profile.

6.3.2.2 Iodination of freeze-thawed granule membranes

The radioactive profile of reducing gels containing granule membranes iodinated by lactoperoxidase after freeze-thawing (storage at -20°C) (see Fig.6.7 and column 4 of Tables 6.6 and 6.7) is similar to that of granule membranes not subjected to freeze-thawing before labelling (Fig. 6.6). Thus CBB stained bands 24 and 26 are the most heavily labelled components although they are not quite so heavily iodinated as they were in freshly labelled granule membranes. Two broad radioactive regions are seen stretching from CBB stained bands 11 to 19 and between bands 35 and 36 to band 40. Band 49 is relatively poorly iodinated whilst band 3, 6, 29, 31, 41-42, 43, 46, 50 and 51 are also labelled.

Differences in the labelling profiles of freeze-thawed and fresh granule membranes shown in Fig. 6.7 and 6.6 respectively, apart from slight differences in the relative incorporation of radioactivity into certain bands (24, 26 and 51), were confined to the region of CBB stained

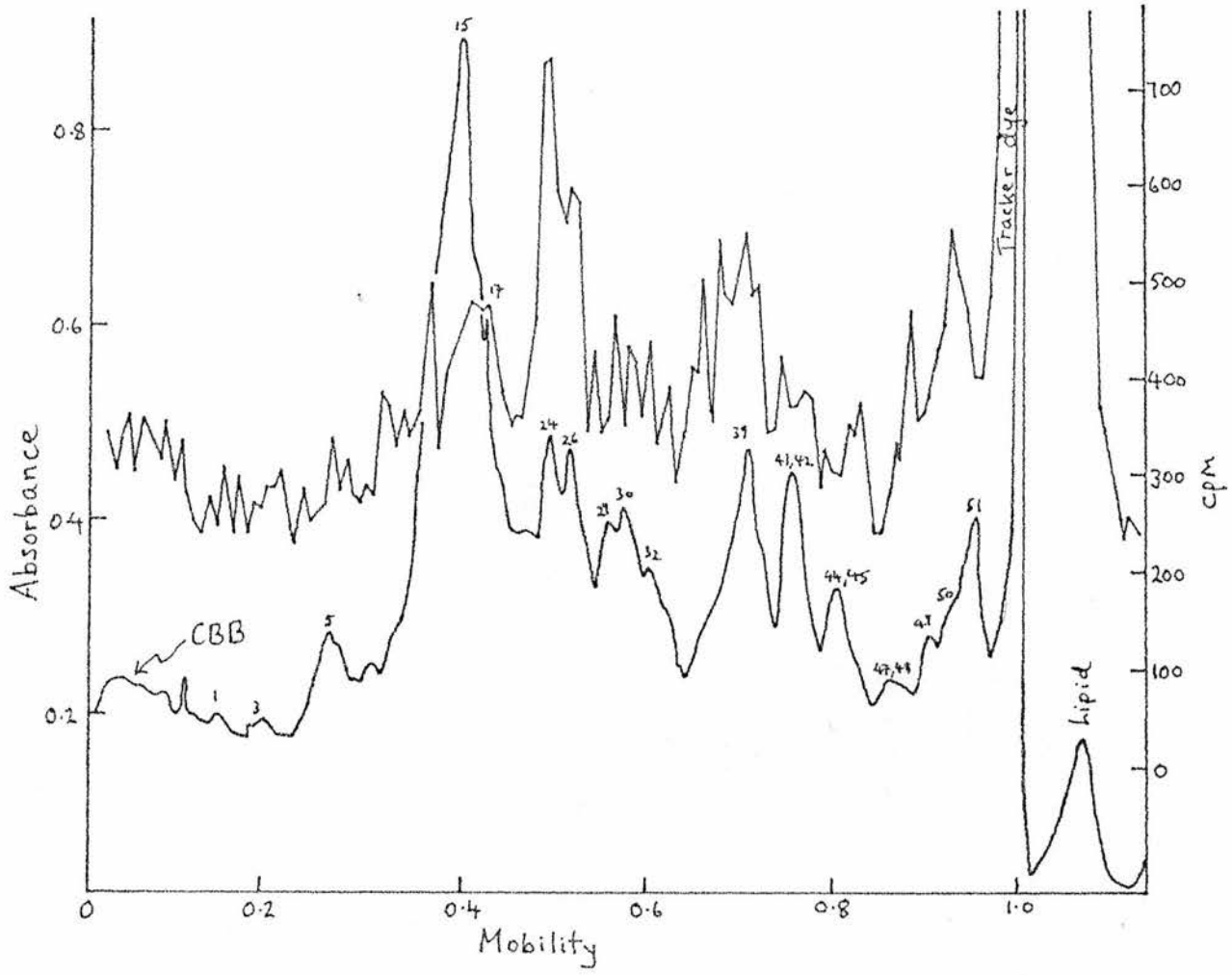


Fig. 6.7 Distribution of Radioactivity in Reducing Gels of Granule Membranes Iodinated with Lactoperoxidase After Freezing and Thawing

Granule membranes which had been stored at -20°C after preparation were iodinated with lactoperoxidase in an analogous manner to that described for freshly prepared membranes (see Fig. 6.6 for further details). SDS PAGE was performed in 8% (w/v) acrylamide gels under reducing conditions, protein was detected with CBB and radioactivity localized by slicing and gamma counting.

bands 28 to 35 and to small differences in the labelling of certain minor staining bands. Thus, in freeze-thawed granule membranes, bands 32 and 34 are labelled whereas bands 28 and 33 are not labelled; minor stained bands 5, 47 and 48 are unlabelled. The results of two other experiments in which freeze-thawed granule membranes were iodinated by the lactoperoxidase method are included in Tables 6.6 and 6.7 (columns 2 and 3). The variability between these is comparable to that between the profiles shown in Figs. 6.7 and 6.6. For instance in experiments 2 and 3 of Tables 6.6 and 6.7 CBB stained bands 24 and 26 are relatively more heavily labelled than in experiment 4. I conclude, therefore that there is no significant difference in the labelling pattern of granule membrane proteins when 'fresh' or 'freeze-thawed' granule membranes are iodinated by the enzyme lactoperoxidase. The following observations are therefore concerned with the general labelling pattern of granule membranes obtained from the results of all experiments.

There is a high radioactive background to reducing gels containing iodinated granule membranes. CBB stained bands 1 and 2, if labelled, are only iodinated to a minor extent. (An exception occurred in experiment 2, when band 2 was very heavily labelled; the reason for this anomaly is unknown). Bands 3 and 6 are labelled in all cases, whereas bands 7,8, 9 and 10 are not usually labelled. In experiments 1 and 3 band 5 is labelled and in experiments 2 and 4 it is not. Further down the gel (towards the anode) a broad radioactive band with sharper bands superimposed on it stretches from CBB stained bands 11 to 20. These sharper radioactive peaks co-migrate with CBB stained bands 11-12, 13, 16 or 17, and 18 and occasionally CBB stained band 15. Between this region and the heavily labelled CBB stained bands 24 and 26 there are normally no radioactive bands except in experiment 3 where band 22 is very heavily labelled and experiment 2 where band 22 is labelled to a

much lesser extent. No clear pattern of labelling emerges for CBB stained bands 28 to 35 although in at least one out of the four cases each particular band is associated with a relatively minor radioactive band. A second broad radioactive band occupies the region of the gel where CBB stained bands 35 to 40 occur. Radioactive peaks superimposed on this broader peak migrate either between CBB stained bands 35 and 36 or with band 36, with band 37, occasionally with band 38 (two out of the four experiments), and with band 39. Band 40 is seen as a shoulder, small peak or not at all in the radioactive profile. In the last section of the radioactive profile bands 41-42, 43, 46, 49, 50 and 51 are all associated with radioactive peaks whilst bands 44, 45, 47 and 48 are probably not labelled. Of the bands 49, 50 and 51 it is the latter two which are relatively heavily labelled.

The broad hump seen in the radioactive profile of gels of reduced granule membranes prepared from intact granules iodinated by lactoperoxidase is also evident in gels of granule membranes iodinated after purification especially in experiments 2 and 3. However, in the latter case, this hump is relatively smaller compared to the peaks superimposed upon it and, as seen above, some of the superimposed peaks are relatively broad themselves.

6.3.2.3 Autoradiography

Autoradiography confirms the similarity of labelling between freeze-thawed and fresh granule membranes (see Fig. 6.2.1 and 6.2.2, lanes 1 and 2, and columns 1 and 2 of Table 6.3).

The autoradiograph also confirms the localization of radioactivity by the slicing and counting method, since membranes used in Fig. 6.6 are the same as those used in lane 2 of Fig. 6.2 and column 2 of Table 6.3 whilst lane 1 of Fig. 6.2 and column 1 of Table 6.3 contain a sample of the same membranes as those used in Fig. 6.7.

CBB bands 1-2 and 4,5 and 6 are both labelled although bands 4, 5 and 6 are better labelled in freeze-thawed membranes whilst CBB stained bands 1 and 2 were not observed to be labelled when the radioactive distribution in gels was determined by the slicing and counting method. In the region occupied by CBB stained bands 11-20 there is a diffusely blackened area in the autoradiogram. Individual bands do seem to be present in this region but it is difficult to localise them and the assignment made in Table 6.3 must be regarded as extremely tentative. Radioactivity associated in the region occupied by CBB stained bands 24 and 26 is clearly confined to these two bands, the radioactive bands being very sharply resolved like the CBB stained bands. In 'fresh granule membranes' a weak radioactive band stretches from approximately CBB stained bands 29 to 33. Although this band is present in 'freeze-thawed membranes' it appears to be split into two, one band co-migrating with CBB stained bands 29 and 30 and the other with CBB stained bands 32 and 33. In both iodinated membrane samples a very broad radioactive band is seen in the region of the gel occupied by CBB bands 36, 37, 38, 39 and 40, although no sharper radioactive bands are seen in this region and this radioactive band appears to stretch further than band 36. Other CBB stained bands which are clearly iodinated are bands 43, 46, 49, 50 and 51 although in the autoradiogram of membranes iodinated after freeze-thawing bands 50 and 51 are not as well separated as they are in the stained gel. CBB stained bands 41 and 42 which run as a single band in these gels are labelled but only to a very minor extent.

The most heavily iodinated band in each profile corresponds to the tracker dye region of each gel. This radioactivity is probably mostly due to free iodide but also includes iodinated peptides which are too small to be resolved from the tracker dye. The broad radio-

active band migrating ahead of the tracker dye is due to iodinated lipids of the granule membrane. As mentioned earlier, since lipid is not 'fixed' in the gel the amount of lipid labelling indicated in the autoradiogram must be regarded as a minimum. (see Table 6.10)

6.3.2.4 Non-reducing gels

An aliquot of the membranes iodinated immediately after preparation (see Fig. 6.6 and column 1 of Tables 6.6 and 6.7)was also subjected to SDS gel electrophoresis under non-reducing conditions. Fig. 6.8 shows the radioactive profile, obtained by 'slicing and counting', and the densitometric scan of the CBB stained gel. The two profiles have been compared in Tables 6.4 and 6.5.

The radioactive profile in non-reducing gels is very different and much simpler than the corresponding profile in reducing gels. Much radioactivity remains at the top of the gel under non-reducing conditions of electrophoresis. Apart from this the largest radioactive band associated with protein occurs at non reduced CBB stained band 7 which is the dimeric form of DβH. However DβH is still poorly labelled in relation to its CBB staining intensity. The experiment clearly shows, however, that, unlike the situation with intact granules, when purified granule membranes are iodinated by the lactoperoxidase procedure, dopamine-β-hydroxylase becomes labelled. Nevertheless, radioactivity is still associated with the region of the gel which DβH occupies under reducing conditions of electrophoresis, due to the presence of iodinated non-reduced CBB stained bands 13, 14 and 15 which presumably contribute to the radioactive peak associated with the fully reduced form of DβH in reducing gels of the labelled membranes.

Non-reduced CBB stained bands 18, 19 and 20 and 21 are all labelled, in a similar proportion to their CBB staining ability. The

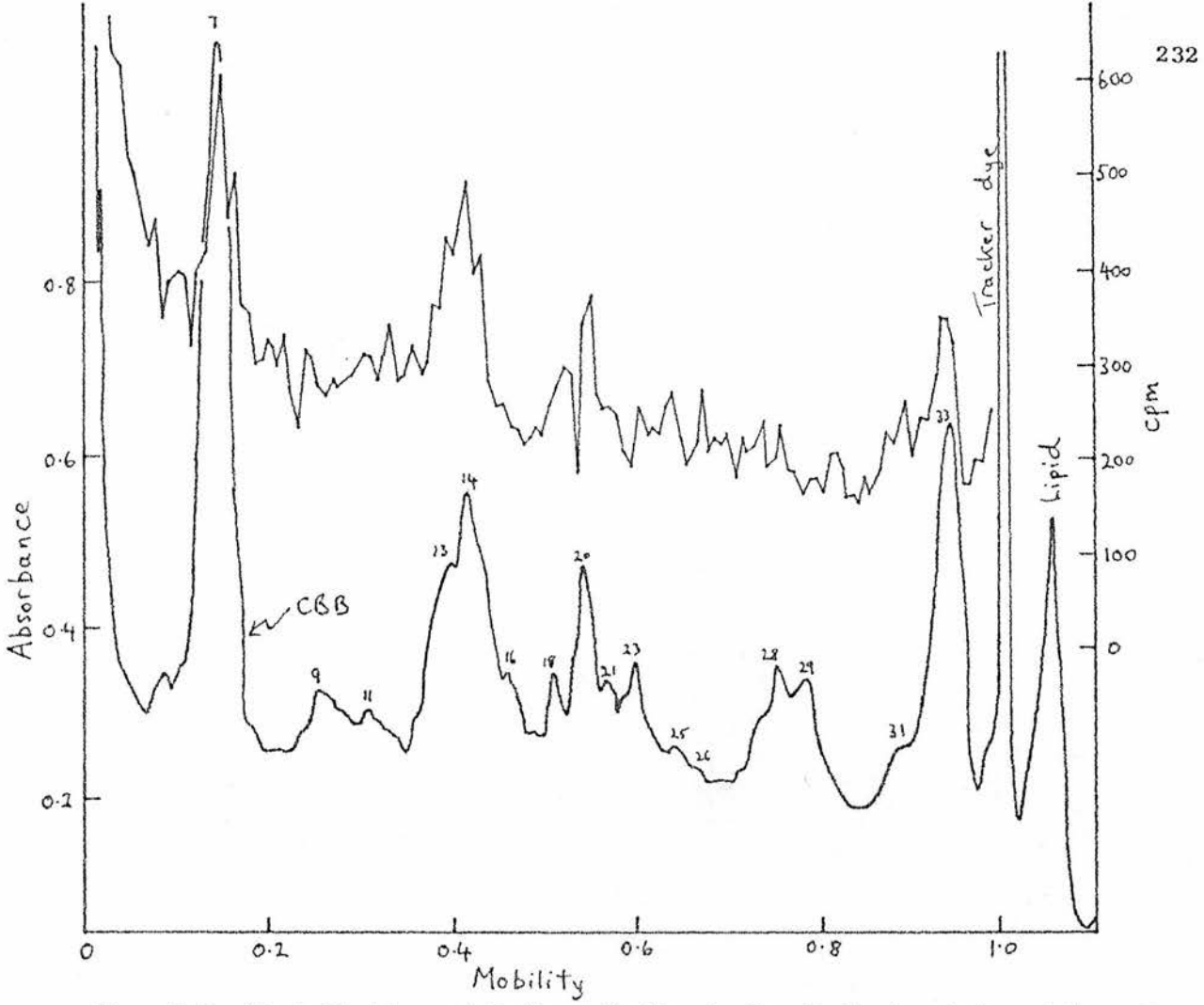


Fig. 6.8 Distribution of Radioactivity in Non-Reducing Gels of Granule Membranes Iodinated with Lactoperoxidase Immediately After Purification

Freshly prepared granule membranes were iodinated with lactoperoxidase as described in Fig. 6.6 and were then subjected to SDS polyacrylamide gel electrophoresis in 8% (w/v) acrylamide gels under non-reducing conditions. Protein is indicated by the densitometric scan of the CBB stained gel at 570 nm and the radioactive profile obtained by slicing the gel into uniform sections (c 1 mm thick) and counting by gamma spectrometry.

relatively major staining area due to non-reduced bands 27, 28, 29 and 30 is, however, not labelled at all. Failure of non reduced band 30 to be labelled is difficult to explain since it is, in terms of mobility, supposed to represent reduced band 46 of the membranes and this band is clearly labelled when the same batch of iodinated membranes is electrophoresized under reducing conditions. At the bottom of the gel non reduced bands 31, 32 and 33 are all iodinated. These bands correspond to reduced bands 49, 50 and 51; non reduced band 31 is, however, less well labelled compared to non reduced bands 32 and 33 than reduced band 49 is compared to reduced bands 50 and 51. It has been suggested earlier that band 49 is composed of two polypeptides both capable of being labelled, but only one of which is represented by non reduced band 31. The tracker dye and lipid regions of non reduced gels of directly iodinated granule membranes are also labelled.

Other interesting features of the non-reducing radioactive profile of granule membranes are the total absence of the radioactive peaks associated with reduced bands 24 and 26 and 36 to 40 when these membranes are run on reducing gels. Despite the disappearance of these radioactive bands from their usual positions no new radioactive bands that could possibly be due to these peaks in the non reduced forms are seen (The only 'extra' radioactive band seen in non-reducing gels compared to reducing gels is that associated with non reduced band 7, the dimeric form of DβH and this was presumed to have arisen from radioactivity associated with bands 13, 14 and 15 in the reduced gels). Therefore radioactivity associated with reduced bands 24, 26 and 36-40, and presumably the polypeptides themselves have presumably failed to enter the gel.

The fact that the radioactivity profile of non reduced membranes is much simpler and shows the absence of certain major iodinated com-

ponents compared to the radioactivity profile of the reduced membranes supports the view that the use of reducing gels provides a fuller, more consistent picture of the labelling patterns of the membranes when they are iodinated whether in the granule or membrane state.

6.3.2.5 Triton X-100 treated membranes

Granule membrane partially solubilized with Triton X-100 prior to iodination with lactoperoxidase show a slightly different pattern of labelling (Fig. 6.9) to that of untreated granule membranes iodinated with lactoperoxidase (Figs. 6.6 and 6.7) under the same labelling conditions. The membranes were separated by SDS polyacrylamide gel electrophoresis under reducing conditions and radioactivity in the gel localized by the 'slicing and counting' method. The radioactive and CBB staining profiles of Triton X-100 treated membranes are compared by the two methods of analysis previously described, in column 5 of Tables 6.6 and 6.7.

The radioactivity profile of Triton solubilized granule membranes iodinated by lactoperoxidase shows features of both the radioactivity profile of granule membranes labelled in the granule state and the profile of granule membranes labelled directly by lactoperoxidase. As in granule membranes prepared from iodinated granules a broad pronounced hump is seen in the radioactivity profile stretching from approximately CBB stained band 11 to band 44 and CBB stained band 49, a low molecular weight minor CBB staining component, is heavily labelled. CBB stained bands of iodinated Triton solubilized granule membranes, which are also characteristically labelled when granule membranes are labelled, are bands 13, 14, 15, 24 and 26 and the region occupied by bands 36-40. Although the DβH region of the gel is just the most heavily iodinated region,it is still poorly labelled by comparison with its

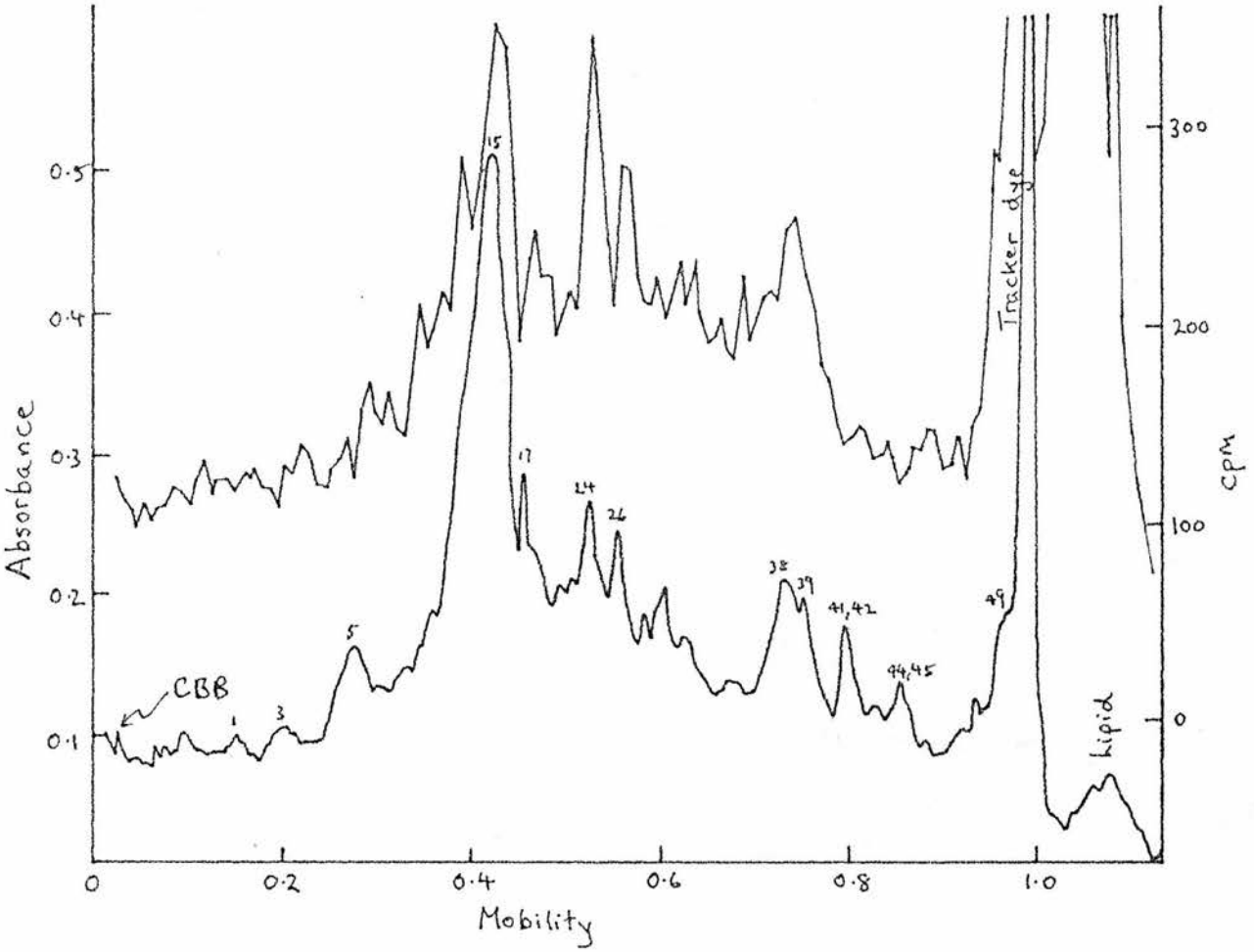


Fig. 6.9 Distribution of Radioactivity in Reducing Gels of Granule Membranes Iodinated with Lactoperoxidase Following Treatment with Tritin X-100

Granule membranes were made 0.1% (v/v) in triton X-100 and then iodinated using lactoperoxidase as described in Fig. 6.6. SDS PAGE was performed in 8% acrylamide gels under reducing conditions. The gel was stained with CBB and the radioactive profile obtained by the slicing and counting method.

staining intensity with CBB, although it is clearly better labelled than when untreated membranes are subjected to labelling. CBB stained bands 36 to 40 are also heavily labelled and together form a broad asymmetric peak in the radioactivity profile. A radioactive peak is also associated with bands 17, 18 and 19. Radioactive peaks which are associated with other CBB stained bands (see Table 6.6, column 5) are relatively small in comparison to those bands described above. Radioactivity is also associated with the tracker dye and lipid regions of the gel.

6.3.3 The labelling of Resealed Chromaffin Granule Ghosts by the LPO/H_2O_2/^{125}I Procedure

The radioactivity profile of granule membranes, prepared from iodinated resealed chromaffin granule ghosts (see section 6.2.5), in reducing gels (Fig. 6.10) bears a close resemblance to that of purified granule membranes labelled directly by lactoperoxidase (Figs. 6.6 and 6.7). Thus CBB stained bands 24 and 26 are by far the most heavily iodinated components; two broad radioactive bands are seen in the region of CBB stained bands 11 to 20 and 35-36 to 40, and there is only a slight hump in the radioactive profile stretching from bands 11 to 12 to bands 44 and 45 approximately. However, band 49 is relatively more heavily labelled than in membranes (as it is in intact granules), and band 50 is also occasionally heavily iodinated.

In Tables 6.8 and 6.9 the results of the labelling of granule ghosts shown in Fig. 6.10 (column 2) together with those of two other experiments are presented. The CBB staining and radioactive profile in each gel have been compared by the two methods previously described in each Table. In each experiment the same general pattern of label-

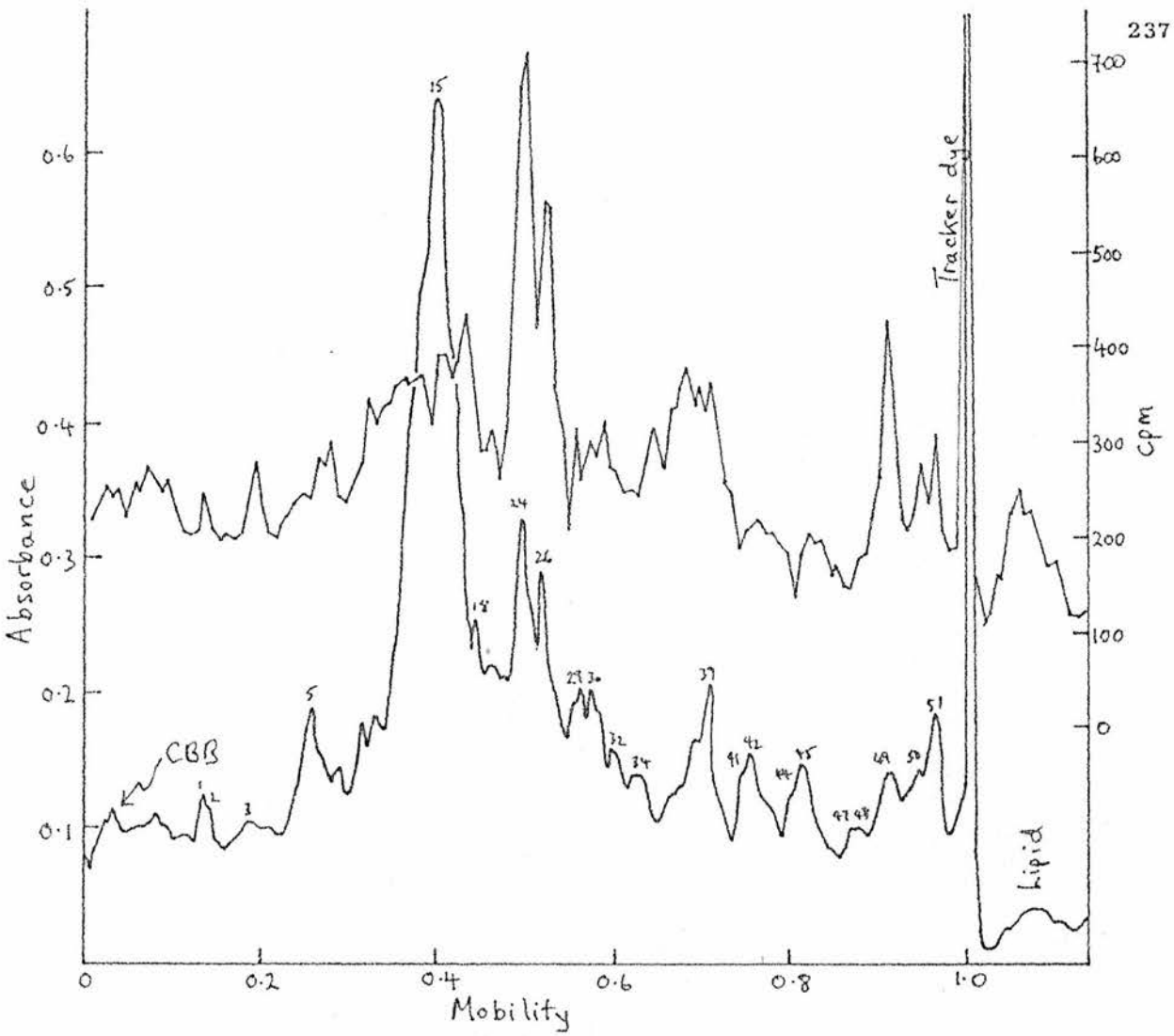


Fig. 6.10 Distribution of Radioactivity in Gels of Resealed Granule Membrane Ghosts Iodinated with Lactoperoxidase.

Resealed membrane ghosts were prepared according to Phillips (1974a, 1977). 1 ml of the ghosts containing 0.5 mg/ml protein were iodinated at 12 - 15°C with 100 µCi Na ^{125}I, 0.04 units lactoperoxidase and 0.375 µmoles H_2O_2 added in 15 0.025 µmole aliquots at 4 min intervals. 2 µgm/ml butylated hydroxytoluene was present and the reaction stopped with 1 µl 14.3 M β-mercaptoethanol. After hypotonic shocking and washing the membranes were separated by SDS polyacrylamide gel electrophoresis in 8% acrylamide gels under reducing conditions. The gel was stained with CBB and scanned at 570 nm. Radioactivity was determined in c 1 mm thick slices of the gel by gamma spectrometry.

Band No.	1	2	3	Comments
1				
2		+		
3	+?	+	+	
4			+	
5	+?			
6		+	+	
7		+		
8		+	+	
9				
10			+	
11			++	
12	+	+	++	
13	+	++	++	DβH
14	+	++	+	
15	++	++	++	
16	++	++	+++?	
17	++	++	+++?	
18	++	+	++++ +?	
19	+	nr	+?	
20	nr	nr	++?	
21	nr	nr	+?	
22	+	+	+?	
23	+	nr	++?	
24	+++++	+++++	++++?	
25	+++	++	+++?	
26	++++	++++	+++++?	
27	++++	+	++++?	
28	+		nr?	
29	+	+	nr?	ATPase subunits
30	++		nr?	
31	++	+	nr?	
32	+	+	+++?	
33	+		+?	
34			++?	
35			+?	
36	+	+ +	+++ ++	
37	++	++	+	
38	++++	++	+++?	
39	+++	++	++	
40	++	+	+	
41				
42		+		
43	+?	+	+	
44				ATPase subunit
45			+	
46	+?	+	+	
47				
48	+?			
49	++	+++	+++	
50	+	+	+++++	CHROMOMEMBRIN B
51	++	+	nr	

Table 6.8 Lactoperoxidase Catalyzed Radioiodination of Resealed Ghosts I

Band No	1	2	3	Comments
1	-	+?	?	
2	-			
3	+	+	+	
4	+ -	s?	+	
5	+	s?	+	
6	-	+	+	
7	-	+	+	
8	-			
9	nr	-	+	
10	nr	-		
11	+	+	++	
12				
13	+ -	s?	+	DβH
14	-	-	-	
15	+	+	s?	
16	-	-	+	
17	+	+	-	
18	-	-	+++ s?	
19	-	nr	s?	
20	nr	nr	+	
21	nr	nr	-	
22	-	+	?	
23	-	nr	?	
24	+++++	+++++	++++	
25	-	-	?	
26	++++	++++	+++++	
27	-	?	s	
28	-	-	nr	ATPase subunit
29	-	+	nr	
30	+	+?	nr	
31	+?	+	nr	
32	-	s	+	
33	+?	-	-	
34	-	-	+	
35	-	-	-	
36	-	+ -	+ -	
37	+	+	- ?	
38	++	+	+	
39	+	+	+	
40	-	s?	+	
41	-	+	-	
42	-			
43	+		+	
44	-	-	+	ATPase subunit
45	-	-	+	
46	+	+	+	
47	-	-	+?	
48	+	-	-	
49	+	++	+	CHROMOMEMBRIN B
50	-?	+	+	
51	-	+	nr	

Table 6.9 Lactoperoxidase Catalyzed Radioiodination of Resealed Ghosts II

ling is observed (as outlined above for the experiment presented in column 2) and this is basically similar to that of granule membranes iodinated after purification except that band 49 is relatively more heavily labelled when ghosts are iodinated.

In the autoradiogram shown in Fig. 6.2 the labelling patterns of granule membranes iodinated in ghosts (lane 3) or after purification from granules (lanes 1 and 2) can be directly compared. The patterns are identical except for heavier labelling of CBB stained band 49 of reduced granule membranes.

6.3.4 Control Experiments

6.3.4.1 Labelling of proteins of the granule lysate in the presence of catecholamines by the LPO/H_2O_2/$^{125}I^-$ procedure

The granule lysate was prepared by hypotonic lysis of 1.8 M sucrose sedimentable granules and removal of insoluble material by centrifugation at 150,000 x g for one hour. A 1 ml aliquot of the supernatant was iodinated with $^{125}I^-$ by the lactoperoxidase procedure as described in the 'Methods' section and the proteins of the lysate were separated by SDS polyacrylamide gel electrophoresis under reducing conditions. Radioactivity within the gel was detected by the 'slicing and counting' method. Fig. 6.11 shows the radioactive labelling pattern of proteins of the granule lysate iodinated in the presence of endogeneous catecholamines.

Incorporation of ^{125}I into protein of the granule lysate is very poor. With the exception of the tracker dye region of the gel, the most radioactive gel slice contains less than 100 cpm and most of the gel slices contain only background amounts of radioactivity. Only one radioactive peak is seen in the profile and this co-migrates with the

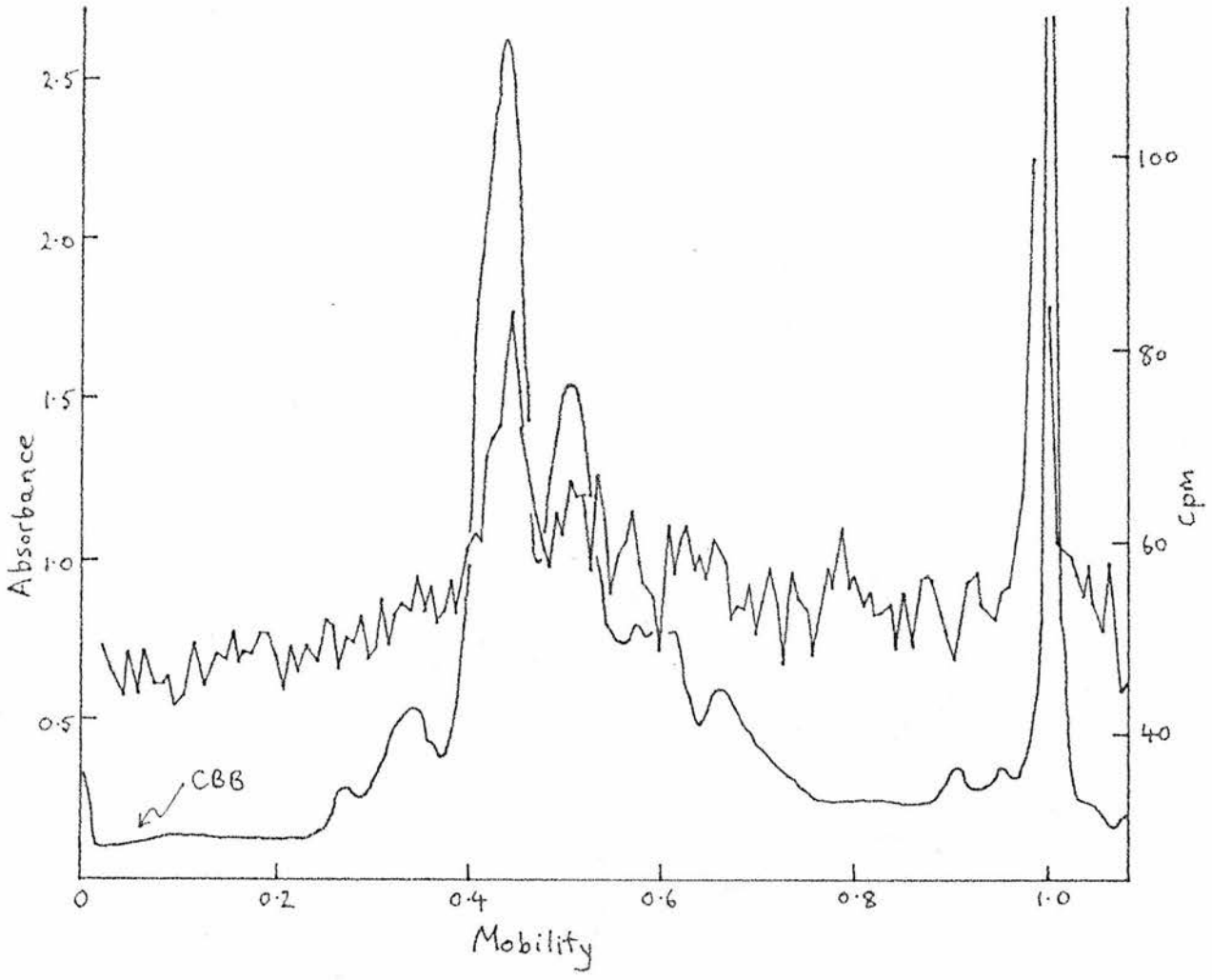


Fig. 6.11 Lactoperoxidase Catalyzed Iodination of the Soluble Proteins of the Chromaffin Granule in the Presence of Endogeneous Catecholamines

Purified chromaffin granules were lysed by hypotonic shock and insoluble material removed by centrifugation at 160,000 g -av for 1 h at 2°C. 1 ml of the supernatant (2 mg/ml protein) was then iodinated with lactoperoxidase as described in Fig. 6.1 except that the reaction was performed at room temperature. The iodinated lysate was then chromatographed on a small Sephadex G-25 column equilibrated in 10 mM hepes, pH 7 in order to remove iodinated catecholamine and free iodide. SDS polyacrylamide gel electrophoresis was performed under reducing conditions in 8% acrylamide gels, protein was visualized with CBB and radioactivity localized by gamma spectrometry of uniform slices of the gel.

CBB stained band representing the protein chromogranin A which is the most heavily stained component in the staining profile of granule lysate proteins. (Other peaks may be present in the tracker dye region of the gel but counts in this region are probably due to free iodide).

The low amount of labelling of the proteins of the granule lysate is probably due to the presence of catecholamines at high concentrations in the reaction mix. Because of their phenolic structure they are theoretically capable of being catalytically iodinated by lactoperoxidase.

6.3.4.2 Iodination of the chromaffin granule membrane in the absence of the enzyme, lactoperoxidase.

Granule membranes were iodinated by the usual procedure (see Materials and Methods) except that lactoperoxidase was omitted from the incubation mix. After washing, an aliquot of the membranes was subjected to SDS polyacrylamide gel electrophoresis under reducing conditions. After staining and destaining, radioactivity in the gel was localized by the 'slicing and counting' procedure (see Fig. 6.12).

Although radioactivity was associated with the top and tracker dye regions of gels of reduced membranes labelled in the absence of lactoperoxidase no radioactive peaks could be seen in the profile. The background count was, however considerably higher than the 'natural' blank background (about 40-50 cpm). As presumed before, radioactivity co-migrating with the tracker dye, bromophenol blue is probably due to free iodide. However, no explanation can be tendered for the significance of the high background and radioactivity associated with the top of the gel.

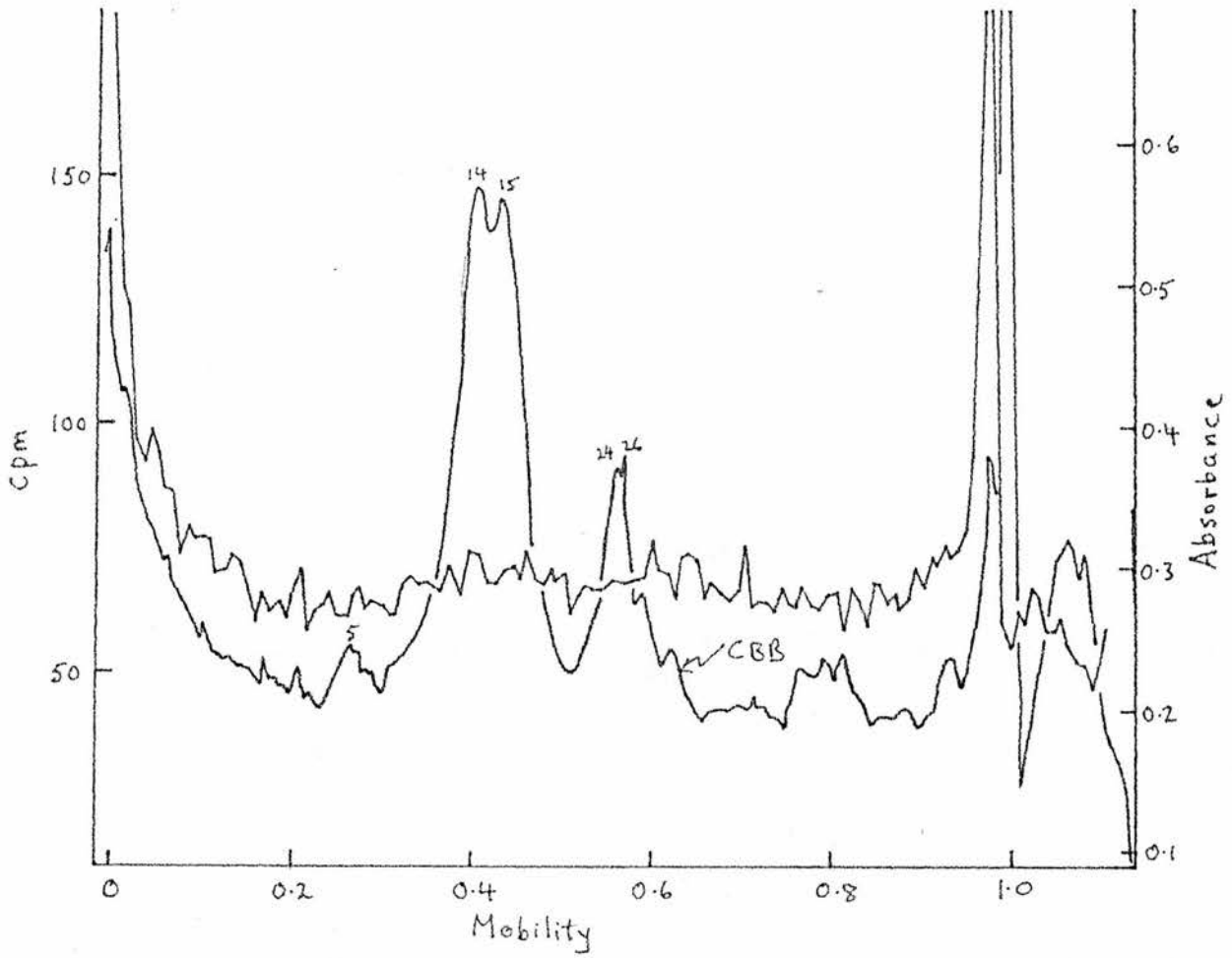


Fig. 6.12 Iodination of Granule Membranes in the Absence of Lactoperoxidase

Granule membranes in 10 mM hepes, pH 7 (1 mg/ml protein), previously stored frozen at -20°C were incubated at room temperature with 100 μCi Na ^{125}I and 2 μgm butylated hydroxytoluene. Hydrogen peroxide (0.375 μmoles) was added in 0.015 μmol aliquots at 4 min intervals for 1 h. The reaction was stopped with 1 μl 14.3 M β-mercaptoethanol and, after washing, the membranes were separated by SDS polyacrylamide gel electrophoresis in 8% acrylamide gels under reducing conditions. The gel was stained for protein with CBB, scanned at 570 nm and then sliced into 1 mm thick sections which were counted in a gamma spectrometer.

6.4 Discussion

6.4.1 Critique of the Lactoperoxidase Labelling Method

Chromaffin granule membranes, granules, and resealed ghosts have been successfully iodinated by the lactoperoxidase technique. However, the conditions of labelling were different because it is much more difficult to iodinate intact granules than their isolated membranes. More hydrogen peroxide and lactoperoxidase were used to iodinate granules but the reaction was performed at lower temperatures. Catecholamines, being phenolic compounds, will be catalytically iodinated by lactoperoxidase, the presence of a certain amount in the suspending medium of chromaffin granules,produced by leakage from intact granules or from lysed granules, leads to an inhibition of labelling of the outer surface of the granules. Leakage of catecholamines from granules is temperature dependent.

Chromaffin granules become increasingly fragile upon purification, especially after sucrose density gradient centrifugation which dehydrates them, and resuspension in isotonic media usually results in substantial granule lysis. Granules suspended in 1.8 M sucrose could have been iodinated but the dehydrated granules are collapsed and the membrane conformation is likely to be different to that of granules suspended in isotonic media. Therefore, a crude preparation of chromaffin granules has been iodinated in which the bulk of other organelles has been removed by differential centrifugation. However, this preparation of granules still contains substantial amounts of other organelles which, apart from competing with the granules for iodination, may inhibit granule iodination in other ways. For instance mitochondria may compete with lactoperoxidase for hydrogen peroxide since they contain glutathione and glutathione peroxidase, sequester iodide, or

directly inhibit the lactoperoxidase catalyzed iodination reaction. Inhibitors of other peroxidases cannot be used since these also inhibit lactoperoxidase. Hence higher enzyme concentrations and more hydrogen peroxide have been employed.

Butylated hydroxytoluene has been used to prevent lipid peroxidation (Welton and Aust, 1972) and addition of hydrogen peroxide in aliquots has been preferred to enzymic generation of hydrogen peroxide. Glucose oxidase will act as an iodinatable substrate for lactoperoxidase and thus compete for iodination with chromaffin granules. It may also be confused with an iodinated membrane protein if the membranes are insufficiently purified. These problems may also arise with lactoperoxidase, itself, or any of the contaminating proteins in the two partially purified enzyme preparations.

However, Tsai et al. (1973) have shown that deviations from the labelling conditions of Phillips and Morrison (1971a) may result in non-vectorial labelling. Under certain conditions, haemoglobin could be iodinated in intact red blood cells by the lactoperoxidase technique whilst bovine serum albumen sequestered in dialysis tubing, which is many times the width of biological membranes, could be iodinated by externally added lactoperoxidase. This is probably due to lactoperoxidase catalyzed oxidation of iodide to iodine. Iodine can cross biological membrane and react sponaneously with phenolic compounds. Experiments which report lipid labelling are probably performed under iodine generating conditions since iodine can insert across double bands present in the fatty acyl side chains. Alternatively iodine could be present in the iodide solution used for radiolabelling.

Labelling of chromaffin granules results in incorporation of iodide into lipid. This is probably due to the generation of iodine

by lactoperoxidase rather than its presence in the radioactive iodide solution since little iodide is incorporated into granule membranes when they are labelled in the absence of lactoperoxidase. There are, however, several facts which seem to indicate that vectorial labelling of the membrane proteins in intact granules does occur when granules are iodinated with lactoperoxidase. Differences exist in the labelling pattern of the membrane proteins when intact granules or isolated membranes are iodinated dopamine-β-hydroxylase, an enzyme known to be exposed at the inner surface of the membrane (Konig et al., 1976; Laduron, 1975) is only labelled in isolated membranes. Granule lysate proteins prepared from iodinated granules are not significantly labelled. (This could be due to the presence of high concentrations of catecholamines but they should also inhibit labelling of the inner surface of the granule membrane).

If one assumes, then, that some degree of vectorial labelling is achieved because catecholamines inhibit labelling at the inner surface of the granule membrane by iodine generated by lactoperoxidase then the labelling profile of membrane proteins iodinated in intact granules will represent proteins buried in the membrane as well as those exposed on the outside of the granule. It is difficult to decide, however, how far into the membrane proteins will be labelled. All internal protein does not appear to be labelled since use of triton X-100 to disrupt granule membranes prior to labelling increases the amount of labelling in certain proteins, especially dopamine-β-hydroxylase. Thus the integrity of the membrane does seem to affect labelling and it may be that lipid is preferentially labelled by iodine. Lipid is in greater concentration in the membrane than protein.

Lactoperoxidase would be expected to have a similar mobility to fully reduced dopamine-β-hydroxylase when subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis. Thus contaminating traces of self-iodinated lactoperoxidase (David, 1972) could be mis-interpreted as labelling of dopamine-β-hydroxylase. However, these proteins will be clearly separated under non-reducing conditions of electrophoresis and labelling of dopamine-β-hydroxylase can be unequivocally determined.

6.4.2 Detection of Radioactive Bands in Polyacrylamide Gels

Radioactivity in polyacrylamide gels was localized by uniformily slicing the gels and counting such slices in a gamma spectrometer. Autoradiography was also used; the low incorporation of radioactivity into the membranes, however, meant that very long exposure times were required. Advanced techniques such as fluorography and the use of intensifying screens were not available, and so autoradiography was not employed routinely. It was, however, used to confirm the localization of radioactivity by the gel slicing method, as shown in Fig.6.2.

Although the gel slicing method was the preferred one, several problems do exist when employing this technique. Resolution of radioactive peaks is obviously dependent on the width of the gel slices. In this work slices were approximately 1 mm thick; this is thicker than many of the CBB stained bands. Hence any radioactivity detected in a particular gel slice may be due to one or several of the bands present in the gel slice. This effect also tends to broaden and flatten radioactive peaks to such a degree that, in certain cases, they may not be distinguished from the background.

It is clearly important to cut slices strictly parallel to the bands. This is particularly difficult when disc gels are used,since the bands are often curved, but the problem also arises with slab gels.

Similarly, the slices must be cut at right angles to the plane of the gel (see Fig. 6.13). In both situations, unlabelled bands near labelled ones may appear to be labelled, owing to the inclusion of a small proportion of the iodinated band in the sliced material which is counted. The possibility of such artefacts makes it especially difficult to decide whether 'shoulders' on major iodinated bands are also labelled.

Another problem is that non-uniformily sized gel slices will lead to a variable background count throughout the gel and will make analysis of minor radioactive peaks that much more difficult. When using the 'gel slicing apparatus' it was found that it was important to keep the gel moist to prevent it from sliding during cutting and to apply even pressure along the whole length of the slices in order to minimize the artefacts described above. If pressure is only applied to the middle of the slices the outer blades tended to splay outwards, cutting at an angle and producing slices of greater width.

6.4.3 Sidedness of Granule Membrane Polypeptides

Using lactoperoxidase catalyzed radioiodination of intact chromaffin granules I find that granule membrane polypeptides represented by band numbers (reduced) 3,4,5,7,8,13,14,24,26,30,31,36,39,40,43,46,49,50 and 51 are consistently labelled. Other bands which are occassionally labelled in granules are bands 6,9,11,12,16,17,18,19,22,23,25,27,28,29,33,35,37,41, 42 and 45. Analysis of dichloromethane extracted granule membranes prepared from iodinated granules confirms that bands 28 and 29, components of the Mg^{++} ATPase, are labelled. Thus the majority of the polypeptides of the chromaffin granule membrane are exposed on the surface of the granule. Band 49, a minor CBB staining component of the membrane and which migrates in the chromomembrin B region of the gel pattern of the membrane, is the most heavily labelled component. A comparison of reducing and non-reducing

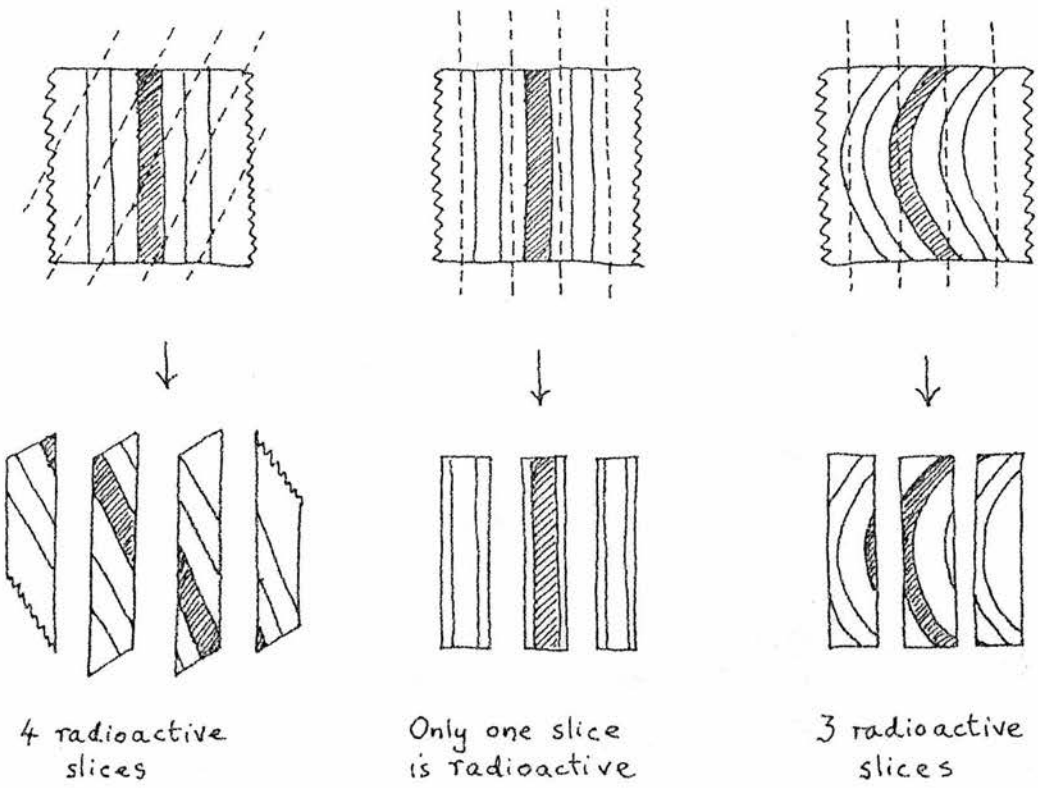


Fig. 6.13 Gel Slicing Artefacts

If slicing is not performed parallel to the protein bands shoulders may be produced upon radioactive peaks in the radioactivity profile of the gel. A similar situation can arise if slicing is performed exactly at right angles to the surface of the gel but that the protein bands are curved.

gels indicates that two or more polypeptides may be contributing to the radioactivity associated with band 49. Other components of chromomembrin B (bands 50 and 51) are also labelled in granules. Dopamine-β-hydroxylase, the major CBB staining component of the granule membrane was consistently unlabelled in the granule labelling experiments, a feature clearly illustrated by running non-reducing gels.

Polypeptides exposed only at the inner surface of the membrane or at both membrane faces can usually be recognised by comparing the labelling patterns of isolated membranes and membranes prepared from labelled granules. However, any conclusions drawn from such a comparison must be tentative for the following reasons: the labelling conditions were submaximal (as well as being different for isolated membranes and intact granules); the conformation of the membrane may have altered in the conversion of granules to broken membranes; and the possibility that exposed regions of polypeptides may not become labelled because of the absence of target groups for lactoperoxidase. Thus labelling of DβH in the isolated membrane but not in the intact granule is only consistent with the view that DβH is located exculsively at the inner surface of the membrane. Similarly one can only suggest that the relative increase in labelling of bands 24 and 26 when membranes instead of granules are catalytically iodinated indicates that these polypeptides are transmembraneous. (Indeed the increased labelling may be due to a co-migratory polypeptide which is only labelled in the membrane state). Band 38, like DβH, may also be an example of a polypeptide located only at the inner surface although labelling seen in this region may be due to glycoprotein (see chapter 4). The relative decrease in labelling of band 49 (compared with bands 50 and 51 especially) may indicate that the conformation of the membrane has altered although other interpretations, such as an increased labelling of bands 50 and 51, are also possible.

Resealed membrane ghosts were conceived as a model system for granule labelling experiments. Because of the absence of catecholamines, lactoperoxidase catalyzed radioiodination of resealed ghosts should be much easier than with intact granules allowing conditions similar to those for granule membrane labelling experiments to be used. However, the labelling pattern of resealed ghosts clearly resembles that of isolated membranes (compare labelling of bands 15, 24 and 26) rather than that of intact granules. This may be because the majority of the ghosts are resealed inside out, or, more likely, that only a fraction of the membrane fragments are resealed. Thus lactoperoxidase catalyzed radioiodination studies of the chromaffin granule membrane reveal that the majority of the polypeptides of the membrane are exposed at the exterior of the granule. Chromomembrin B is particularly well exposed at the outer surface and at least two of the subunits of the Mg^{++} ATPase are also on the outside. Dopamine-β-hydroxylase is probably located at the inner surface only and two components (bands 24 and 26) may be transmembraneous, i.e. exposed at both membrane faces.

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CHAPTER 7

LABELLING OF CHROMAFFIN GRANULES WITH A SMALL MOLECULAR WEIGHT, FLUORESCENT PROBE

LABELLING OF CHROMAFFIN GRANULES WITH A SMALL MOLECULAR WEIGHT, FLUORESCENT PROBE

7.1 Introduction

N-(iodoacetylaminoethyl)-5-napthylamine-1-sulphonic acid (1,5-I-AEDANS, see Fig. 7.1.1) is a fluorescent sulphydryl reagent which was first synthesized by Hudson and Weber (1973). This compound combines the reactivity of iodoacetamide towards sulphydryl groups with the spectral properties of napthalenesulphonic acids to form fluorescent derivatives with sulphydryl compounds (Hudson and Weber, 1973). 1,5-I-AEDANS (and its 1,8 isomer) have been shown to react specifically with sulphydryl groups in proteins (see Fig. 7.1.2), such as globin and papain (Hudson, 1970), rhodopsin (Wu and Stryer, 1972), and myosin (Mendelson et al., 1973). In addition the negative charge conferred by the sulphonic acid group should ensure that 1,5-I-AEDANS is sufficiently hydrophilic to prevent it from easily diffusing across biological membranes. Thus, 1,5-I-AEDANS would seem to possess sufficient properties for it to be used to probe the arrangement of proteins.

In this chapter are reported experiments in which 1,5-I-AEDANS was used to try and identify proteins of the chromaffin granule membrane exposed on the outside of chromaffin granules. Intact granules were incubated with 1,5-I-AEDANS under various conditions before the reaction was terminated by the addition of excess β-mercaptoethanol. Chromaffin granule membranes and granule lysate were then prepared from the labelled granules. Labelled proteins could be detected in polyacrylamide gels containing labelled membranes or lysate separated by SDS polyacrylamide gel electrophoresis by scanning the gels for fluorescence and comparing this scan with the CBB staining profile of the gels. The 'fluorescence

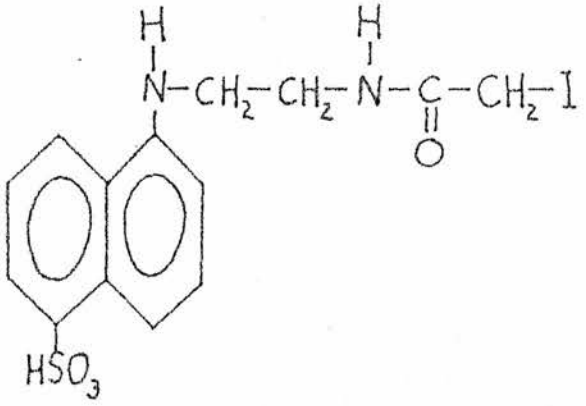

Fig. 7.1.1 1,5-I-AEDANS

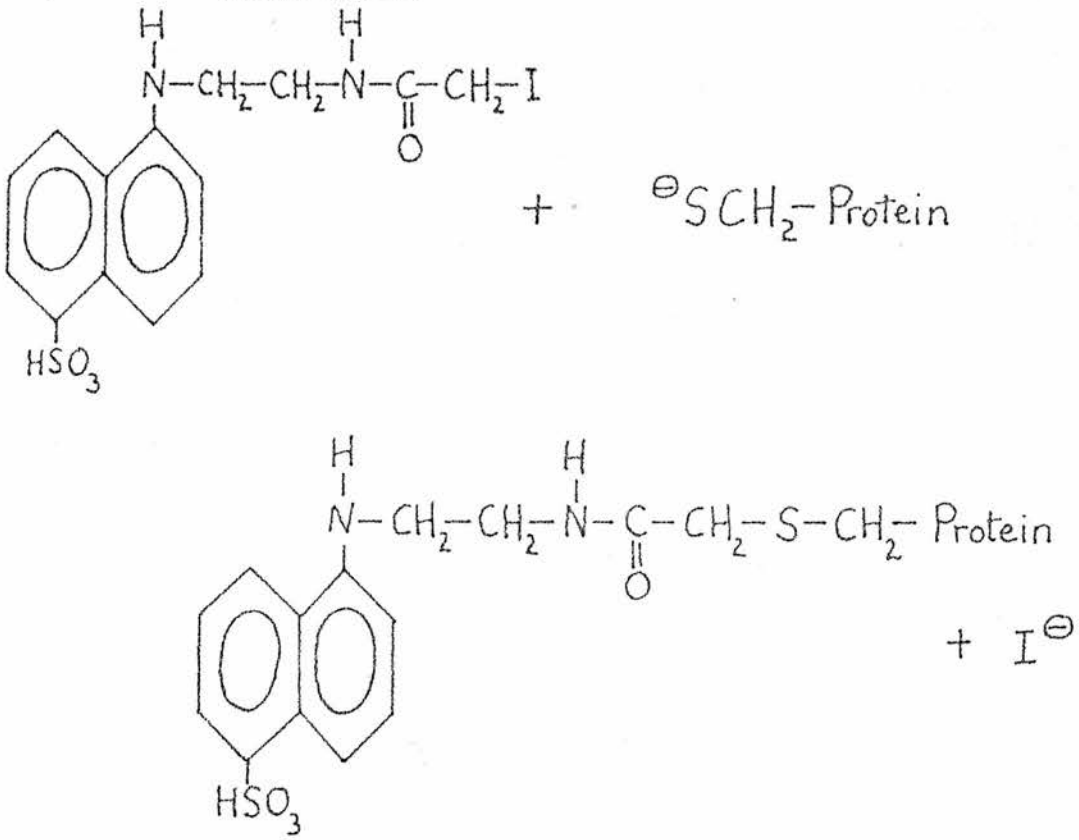

Fig. 7.1.2 Reaction of 1, 5-I-AEDANS with Thiols

scanner' consisted of a suitably modified Gilford linear transport densitometric gel scanner (see section 2.2.3). 'Controls' used to assess the membrane permeability of this reagent included analysis of granule lysate proteins prepared from 1,5-I-AEDANS reacted intact granules for label and comparison of the labelling patterns of membranes labelled in intact granules or as isolated membranes. In the latter case one would expect more proteins to be labelled due to the increased accessibility of the proteins on the inner surface of the membrane to this reagent once the permeability barrier has been disrupted.

A major drawback to the use of 1,5-I-AEDANS is its photocatalyzed degradation in aqueous media (see Hudson and Weber, 1973) which not only renders the compound inactive but also more importantly there is the possibility that the photoactivated excited state may react with various bonds in proteins and lipids in a manner similar to the reaction of diazo derivatives (Shafer et al., 1966; Converse and Richards, 1969). To overcome these problems the labelling reaction and handling of 1,5-I-AEDANS were conducted in the dark or with minimal exposure to light. Once the reaction has been terminated, by the addition of β-mercaptoethanol to react with excess 1,5-I-AEDANS, this precaution can be dispensed with since it is the presence of iodine in 1,5-I-AEDANS which confers its photolability.

7.2 Materials and Methods

7.2.1 Materials

1,5-I-AEDANS was obtained from Aldrich Chemical Co. Ltd., Gillingham, Dorset, U.K. and L-Cysteine Hydrochloride from BDH Ltd., Poole, Dorset,U.K.

7.2.2 Labelling of Intact Chromaffin Granules with 1,5-I AEDANS

The large granule fraction (approx 5 mg/ml protein in 0.3 M sucrose, 10 mM hepes pH 7), prepared as previously described (see section 2.1.1) was reacted with either 1 mM or 3 mM 1,5-I-AEDANS by incubation at room

temperature for 15 minutes or at 4°C overnight (usually 16 hr or more) always in the dark. The incubation was terminated by returning the granules to ice (where appropriate) and by adding excess (c50 mM) β-mercaptoethanol. Granule membranes and granule lysate were then purified from the labelled granules in the standard manner (see section 2.1.2).

7.2.3 Labelling of Freeze-Thawed Chromaffin Granules with 1,5-I-AEDANS

Chromaffin granules,which had been pelleted through 1.8 M sucrose, were suspended in 1 ml 10 mM hepes, pH 7 containing 3 mM 1,5-I-AEDANS and subjected to 5 cycles of freezing and thawing after which they were incubated overnight at 4°C in the dark. At the end of this period excess β-mercaptoethanol was added and particulate material was removed from solution by centrifugation at 100,000 xg for 1 hr (Beckman L2 ultracentrifuge, 65 rotor) at 2°C. The supernatant granule lysate was dialyzed against 10 mM hepes, pH 7 in the cold. The pellet was taken up in a minimum volume of 1.8 M sucrose,10 mM hepes, pH 7 and shocked by dilution with 10 mM hepes, pH 7. Granule membranes were recovered by centrifugation (160,000 xg for 1 hr at 2°C) and the process of hypotonic shocking and membrane recovery by centrifugation repeated twice before the membranes were finally resuspended in 10 mM hepes, pH 7.

7.2.4 Labelling of Isolated Chromaffin Granule Membranes with 1,5-I-AEDANS

Granule membranes (1 mg/ml protein in 10 mM hepes, pH 7) were labelled with 1,5-I-AEDANS under similar conditions to those used to label granules (see above). The labelled membranes were washed 3 times with 10 mM hepes, pH 7 (see section 2.1.2) and stored frozen in the same buffer.

7.2.5 Labelling of Isolated Chromaffin Granule Lysate with 1,5-I-AEDANS

Chromaffin granule lysate, i.e. the supernatant resulting from the centrifugation of hypotonically shocked 1.8 M sucrose pelletable granules, at approximately 2.5 mg/ml protein were incubated with 1 mM or 3 mM 1,5-I-AEDANS overnight at 4°C in the dark. The reaction was ended by the addition of excess β-mercaptoethanol and the lysate dialyzed against 10 mM hepes, pH 7 in the cold.

7.2.6 Analysis of 1,5-I-AEDANS Labelled Samples

SDS polyacrylamide gel electrophoresis of 1,5-I-AEDANS labelled samples was performed under reducing conditions in 8% (w/v) acrylamide slab gels (section 2.2.1). After electrophoresis, the unstained gels were sectioned into individual lanes and placed in 7% acetic acid/5% methanol to await scanning for fluorescence. Individual lanes were scanned for fluorescence in a quartz cuvette containing 7% acetic acid/5% methanol using the modified Gilford linear transport densitometric gel scanner (section 2.2.3), before staining with CBB.

7.3 Results

The gels were slightly 'overloaded' with protein in order to produce good fluorescence scans. In general, however, the CBB staining profiles of gels of 1,5-I-AEDANS labelled granule membranes and granule lysate separated by SDS polyacrylamide gel electrophoresis under reducing conditions in 8% (w/v) acrylamide gels was similar to that previously observed (see chapter 3).

It was found that, after staining and destaining, the gels had swollen and because the densitometric scan of a gel was longer than the

fluorescence scan the two scans could not be directly compared without making some compensation for the difference in size. R_f's (i.e. the distance travelled relative to that of the tracker dye) could not be used since the swelling of the gels did not appear to be uniform. Major fluorescent peaks did appear to correspond to major CBB stained bands, in general, and, because the differences in length of the fluorescence and densitometric scans was not great, sections of the two scans could be directly compared over a small distance by aligning the major peaks in this area of each scan. In this way a detailed comparison of the fluorescence and densitometric scans of the individual lane of a slab gel containing 1,5-I-AEDANS labelled proteins could be made.

7.3.1 The Labelling of Intact Granules with 1,5-I-AEDANS

Granule membranes labelled with 1,5-I-AEDANS in the intact granule were subjected to SDS polyacrylamide gel electrophoresis under reducing conditions in 8% (w/v) acrylamide gels. Fig. 7.2 shows the fluorescence and densitometric scans of such a gel. The two scans have been compared in column 2 of Table 7.1 where CBB stained bands of reduced granule membranes have been awarded between one and five pluses according the the size of the fluorescent peak with which they co-migrate. Other experiments in which granule membranes were labelled with 1,5-I-AEDANS in the granule state are also analyzed in Table 7.1. Because of slight overloading of gels,in order to obtain good fluorescence scans, only major CBB staining bands could be consistently identified.

The fluorescence scan of gels of granule membranes prepared from 1,5-I-AEDANS labelled granules showed many sharp peaks as well as a 'background' throughout the length of the scan and was unaffected by the different labelling conditions used viz.,1 mM or 3 mM 1,5-I-AEDANS and

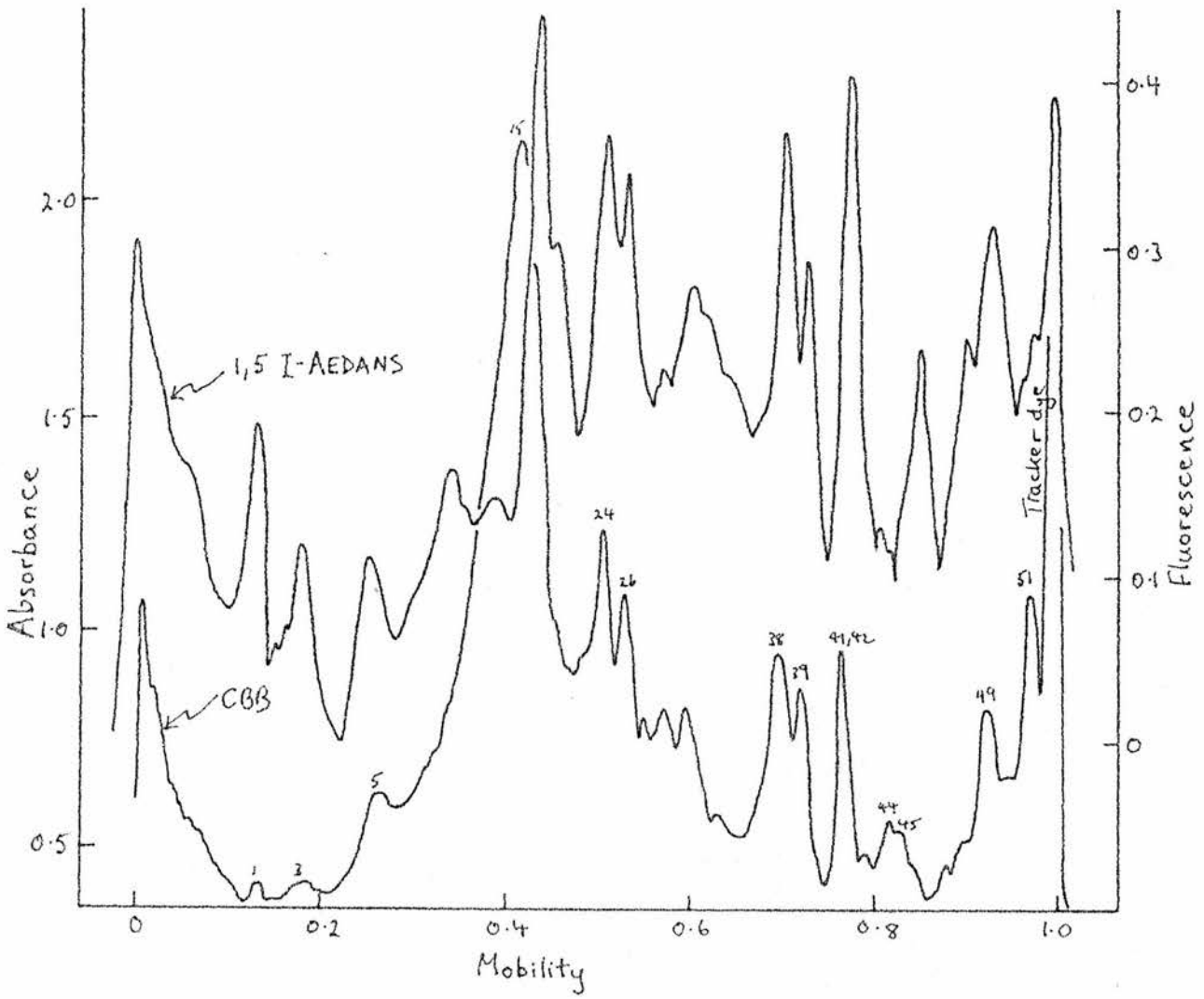

Fig. 7.2 Labelling of Intact Granules with 1, 5-I-AEDANS

Granules were reacted with 3 mM 1, 5-I-AEDANS at 4°C overnight and in the dark. Membranes (150 µgms protein) prepared from the labelled granules were subjected to SDS polyacrylamide gel electrophoresis under reducing conditions in 8% (w/v) acrylamide gels. The gel was scanned for fluorescence prior to staining with CBB for protein and then densitometrically scanned at 570 nm.

Table 7.1 I-AEDANS Labelling of Intact Chromaffin Granules

Fluorescence and CBB staining profiles of gels of granule membranes prepared from intact granules labelled with 1,5-I-AEDANS are compared. In experiments 1 and 3 granules were labelled with 1 mM 1,5-I-AEDANS whereas in experiments 2, 4,5,6 and 7 3 mM 1,5-I-AEDANS was used. The granules of experiments 8 and 9 were treated with 1 mM L-cysteine hydrochloride for 1 h at $0^{o}C$ prior to reaction with 2.5 mM 1,5-I-AEDANS. For details - see text.

Band No.	1	2	3	4	5	6	7	8	9
1	+++	++	++	++	++	+	++	++	+
2									
3	+	+	+	+	+		+	+	+
4				broad					
5	+	+	+	+ broad	+	+	+	+	+
6									
7									
8									
9									
10	+	+	++	+	+		++	+	+
11									
12									
13	++	++	++	+	+		+++	+	+
14	?	?	?	?				?	
15	?	?	?	?				?	
16	+++++	+++++	++++	+++++	+++++	+++++	+++++	+++++	+++++
17									
18									
19	+++	+++	++			+++		++	+ small
20									
21									
22									
23	?	?	?					?	
24	++++	++++	+++++	+++	++++	++++	+++++	++++	++
25	?		?	?				?	
26	++++	++++	+++++	+++	++++	++++	+++	++	++
27	?	?	?	?				?	2 peaks + small
28	?	++	+++s	?				?	
29	?			?				?	
30	?	?	+++s	?				?	
31	?		+++s	?				?	
32	+++	+++	+++	+++	+	++++	+++	+	
33	?	++						+	
34	?	+	+?	+?	+		++ shoulder	?	
35	?	+						?	
36	?	+	?	?	+			?+	
37	?	+	?	?				?+	
38	++	+++	++	++	+++	++	+++	++	++
39	++	+++	?	+	+++	++	+++	+?	?
40	?	?	?	?	+				+
41	++++	++++	++++	+++	++++	+++	+++	+++	++++
42									
43	?	?+	?	?					
44	?	?	+?	+?	+?	?			+?
45	?	?	?	?		?			
46	++	++	++	++	++	++	++	+	++
47	?	?					++		
48	?	+	+				+++	+	
49	++	++	++				+++	++	
50	?	?	?					+?	
51	?	+	?					?	
52			?						
53									

Table 7.1

incubation for 15 mins at room temperature or overnight at 4°C or by using granules pre-incubated with 1 mM L-cysteine hydrochloride for 1 hr at 4°C for reaction with 3 mM 1,5-I-AEDANS.

The following features of the labelling pattern of granule membrane proteins reacted with 1,5-I-AEDANS in intact granules were observed. A fluorescent band was observed at the top of the gel and seemed to be due to labelled protein which failed to or only just entered the gel. Like the CBB stained material this band was extremely variable in intensity and also 'tapered' into the gel. CBB stained bands 1 and 2, very minor staining components migrating as a doublet, were associated with a single moderately intense fluorescent band and are clearly very heavily labelled with 1,5-I-AEDANS. A broad smaller fluorescent peak is associated with CBB stained band 3. Resolution of CBB stained bands in the region of the profile (up to band 15) is poor. Bands 4, 5 and 6 and possibly 7 and 8 co-migrate and this resultant single broadly stained area is associated with a correspondly broad fluorescent band whilst bands 9 to 14 cannot be distinguished from the trailing slope of CBB stained band 15. Several small fluorescent peaks are occassionally observed in this region. CBB stained band 15 (DβH subunit) is the most heavily stained region of the gel of reduced granule membranes but appears to be poorly labelled by 1,5-I-AEDANS under these conditions. An intense fluorescent band, usually the most intense of the profile, is associated with the leading edge of band 15. In contrast to CBB stained band 15 the fluorescent band is always very sharply resolved and because of this and the imperfect alignment it seems likely that this fluorescent band is due to another CBB stained band obscured by the leading edge of band 15. A shoulder is sometimes observed on the leading edge of this fluorescent peak and therefore, there are at least two major labelled components in this region. CBB stained bands 16 to 22

are not resolved and all occur in this region of the gel and one or more of these bands could be labelled to produce this fluorescent band and its shoulder. Two characteristically intense fluorescent bands co-migrate with bands 24 and 26. It is difficult to decide which of the CBB stained bands 28 to 35 are labelled by 1,5-I-AEDANS. The resolution of these bands is extremely poor and, although there are fluorescent peaks associated with this region they are variable in position. Because of the high background in this region the fluorescence is never zero although this could be due to the diffuse CBB stainable background which is also observed. The weight of evidence seems to indicate that CBB stained bands 32 and 33 are associated with a small fluorescent peak. CBB stained bands 38, 39 and 41, 42 are very well labelled. The minor CBB stained peaks 36, 37, 40 and 43 associated with the major stained bands indicated above may also be labelled but only to a minor extent and in proportion to their staining ability compared to bands 38, 39 and the doubted 41-42. A slightly broader fluorescent peak of moderate height is associated with CBB stained bands 44-46 and appears to be centred upon band 46 which is the least heavily stained band of the three. CBB stained bands 48 and 49 are clearly labelled. The shoulders associated with the fluorescent peaks due to CBB stained band 48 and the dye front are probably due to CBB stained bands 47, 50 and 51. Fluorescence associated with the tracker dye is probably due to 1,5 ME-AEDANS, the product of the reaction between 1,5-I-AEDANS and β-mercaptoethanol, or to 1,5-I-AEDANS labelled peptides too small to be resolved from the tracker dye bromophenol blue.

In contrast, the proteins of the granule lysate prepared from 1,5-I-AEDANS labelled granules showed poor incorporation of the label (see Fig. 7.3,1). Thus, under a variety of conditions, the chromaffin

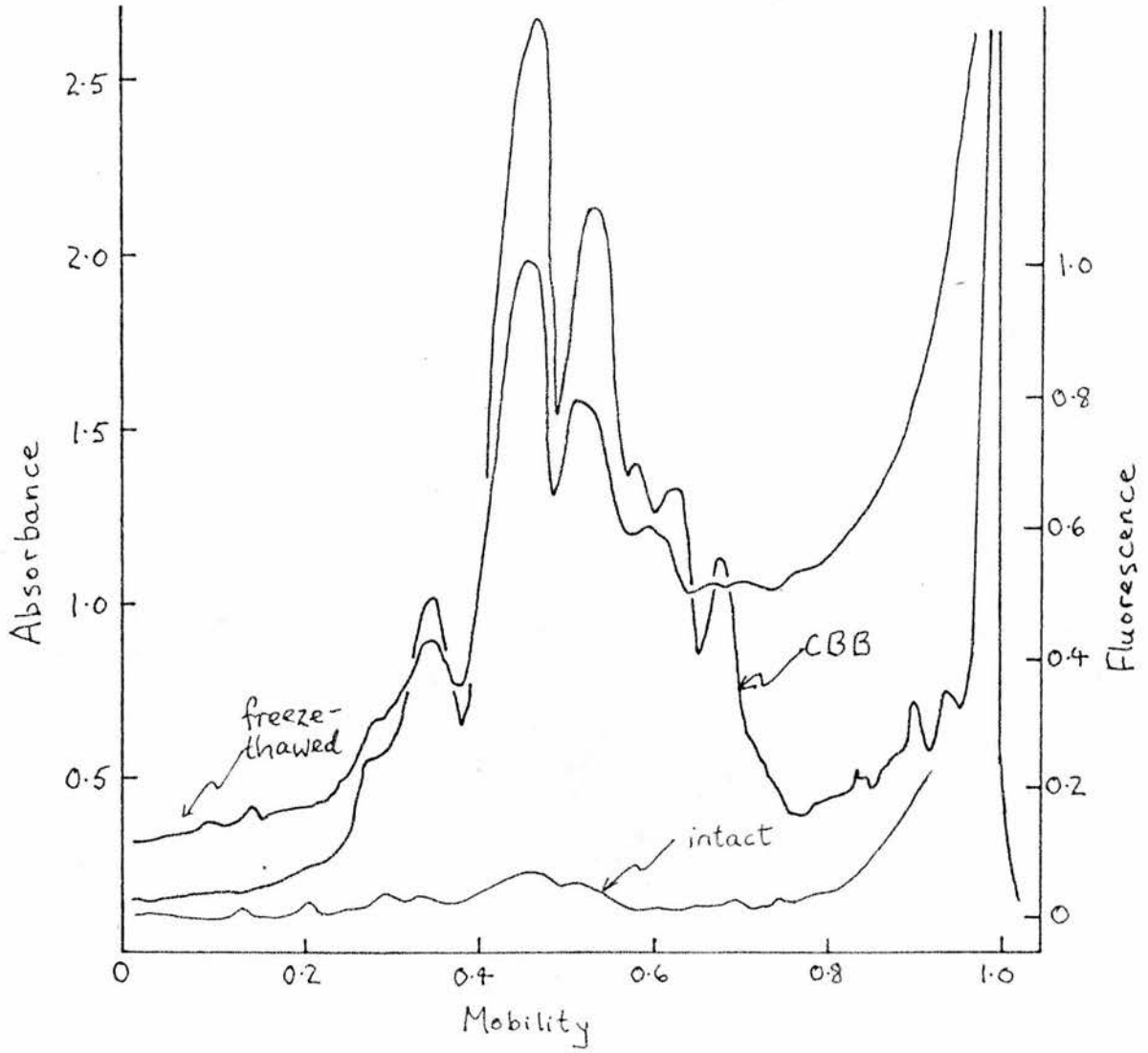

Fig. 7.3.1 Labelling of Granule Lysate Proteins in Intact and Freeze-Thawed Granules with 1, 5-I-AEDANS

Granule lysate prepared from intact granules previously labelled with 1,5-I-AEDANS (see Fig. 7.2) or from freeze-thawed granules labelled with 1, 5-I-AEDANS (see section 7.2.3) was separated by SDS polyacrylamide gel electrophoresis under reducing conditions in an 8% (w/v) acrylamide gel. 1, 5-I-AEDANS was detected by fluorescence scanning and protein by staining with CBB.

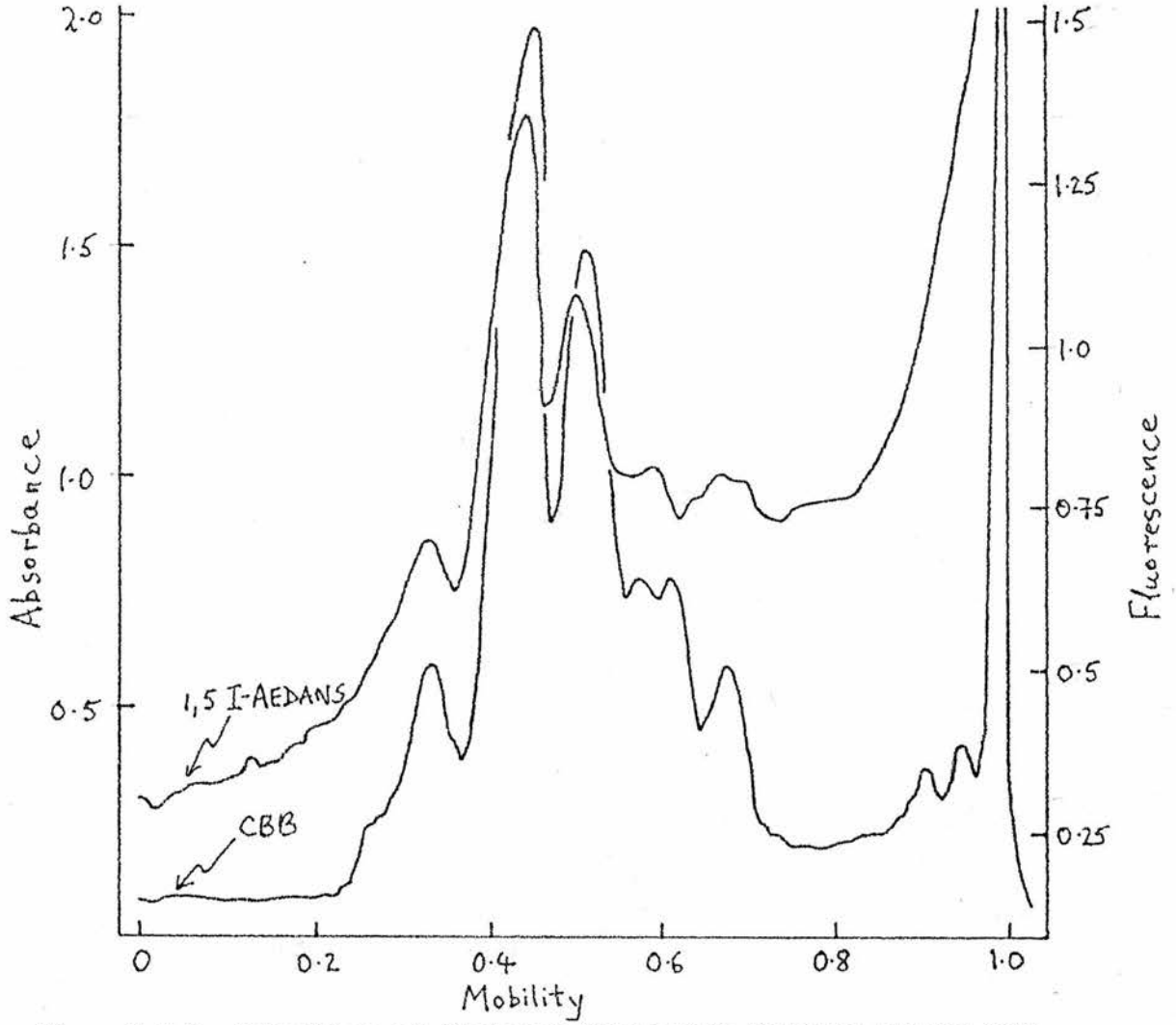

Fig. 7.3.2 Labelling of Isolated Chromaffin Granule Lysate with 1, 5-I-AEDANS

Isolated chromaffin granule lysate (2.5 mg/ml) was labelled with 3 mM 1, 5-I-AEDANS by incubation overnight at 4°C in the dark. SDS polyacrylamide gel electrophoresis was performed in 8% (w/v) acrylamide gels under reducing conditions. Approx. 150 µgm protein was applied to the gel.

granule membrane has been successfully labelled in intact chromaffin granules without significant labelling of the proteins of the granule matrix.

7.3.2 The Labelling of Isolated Chromaffin Granule Membranes with 1,5-I-AEDANS

The fluorescence scan of a gel lane containing chromaffin granule membranes labelled with 1,5-I-AEDANS after isolation is shown in Fig.7.4. This pattern of labelling (Table 7.2) was essentially unaltered whether the membranes were freshly prepared, had been stored frozen at -20^{o}C, were pre-incubated with 1 mM L-cysteine hydrochloride for 1 hr in ice, or were labelled at room temperature (15 or 30 minutes with 1 mM or 3 mM 1,5-I-AEDANS) or at 14^{o}C overnight (with 1 mM or 3 mM 1,5-I-AEDANS).

Fig 7.2 and 7.4 depict typical labelling patterns of granule membranes labelled with 1,5-I-AEDANS in the granule or isolated membrane state respectively and it is immediately obvious that they are essentially identical. Minor differences observed between the two profiles (e.g. differences in the relative intensity of labelling of CBB stained bands 16, 24 and 26, the shape of the fluorescence profile in the region of the profile occupied by CBB stained bands 28 to 35 and the heavier labelling seen in Fig. 7.4 in the region between CBB stained bands 41-42 and 46 of the membrane) are typical of those differences seen when the labelling profiles of granule membranes labelled in one state only are compared with one another. This view is confirmed by the results of the analyses shown in Tables 7.1 and 7.2.

7.3.3 The Labelling of Isolated Chromaffin Granule Lysate with 1,5-I-AEDANS

As can be seen from Fig.7.3.2 the proteins of the chromaffin granule lysate are extremely well labelled when the isolated total lysate

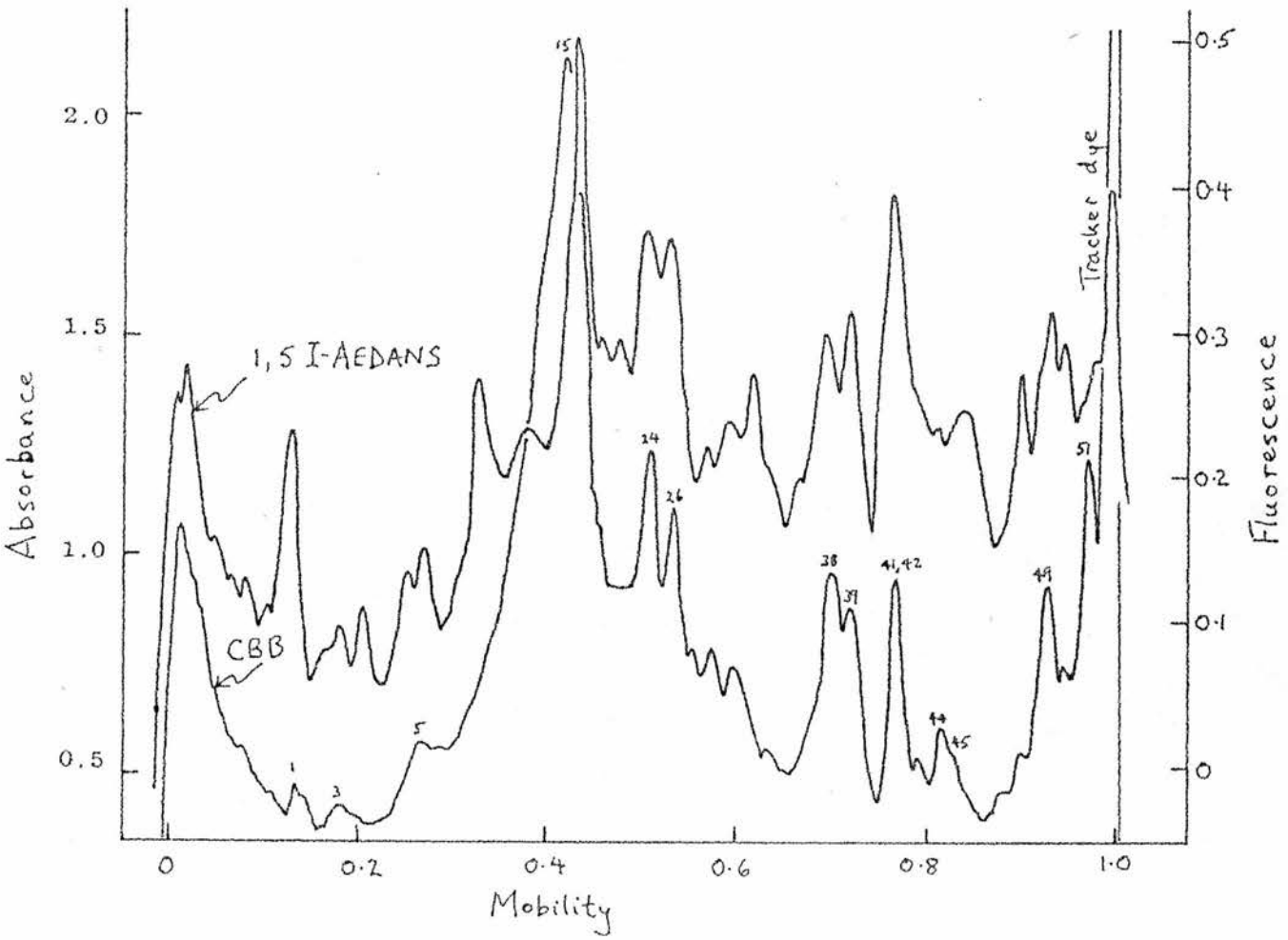

Fig. 7.4 Labelling of Purified Chromaffin Granule Membranes with 1, 5-I-AEDANS

Isolated granule membranes (1 mg/ml protein) were reacted with 1 mM 1, 5-I-AEDANS overnight at 4°C and in the dark. The labelled membranes were separated by SDS polyacrylamide gel electrophoresis (8% (w/v) acrylamide gel, reducing conditions) and detected by fluorescence scanning. Also shown is the CBB staining profile of the gel.

Table 7.2 I-AEDANS Labelling of Isolated Granule Membranes

Fluorescence and CBB staining profiles of gels of granule membranes (reduced) labelled in the isolated state with 1 mM (expt. 1) or 3 mM (expt 3 and 4) 1, 5-I-AEDANS. In experiments 2, 5 and 6 granules purified by sedimentation through 1.8 M sucrose are lyzed in the presence of and then incubated with 1, 5-I-AEDANS (3 mM). Membranes in experiments 7 and 8 were treated with 1 mM L-cysteine hydrochloride for 1 hr at $0^{\circ}C$ prior to reaction with 2.5 mM 1,5-I-AEDANS. For further details - see text.

Band No.	1	2	3	4	5	6	7	8
1	++	++	++	+	+	++	+	+
2								
3	+	+ broad	+	+	+	++	+	
4								
5	+	+ broad	+	+ v. broad	+	++	+	+
6								
7								
8								
9								
10	++	+	+			+	+	+
11								
12								
13	+	+					+	+
14	?					++?	?	
15	?						?	
16	+++++	++++	+++++	+++++	+++++	+++++	+++++	+++++
17								
18								
19								
20	+		+				++	+
21								
22	+							
23								
24	+++	+++++	++++	++++	+++++	+++	+++	++++
25								
26	+++	++++	++++	+++	+++++	+++	+++	+++
27	+s							
28			+					
29			+					
30	+		+				+	+
31	+		+				+	+
32	+	+++	+	↑?	↑	+++	+	+
33	?		+	? ++	+++?			
34	+ s		+	↓?	↓	+	+	+
35	s		+					
36	+						+	
37								
38	++	+++	+++	++	+++	++	++	++
39	++	++	++		+++	++	++	+
40	+?						+	
41	+++	++++	+++	+	++++	+++	+	++
42								
43	+						?	+
44	?s		?				?	
45	?s		?		++		?	
46	+	++	++	+	+++	++	+	+
47							+	
48	+					+++	+	
49	++					+++	+	
50	+						+	
51	+						+	
52								
53								

Table 7.2

(i.e. the total granule contents minus the granule membrane) were reacted with 1,5-I-AEDANS. All the major CBB stained bands of the lysate (reduced lysate bands 2,3,5,7,8,9 and 10) were labelled and roughly in proportion to their ability to stain with CBB. The reaction conditions were similar to those used to label intact chromaffin granules and isolated chromaffin granule membranes. Therefore if 1,5-I-AEDANS does enter the matrix of intact chromaffin granules one would expect its presence to be detected by labelling of the proteins of the granule lysate.

7.3.4 The Labelling of Freeze-Thawed Chromaffin Granules with 1,5-I-AEDANS.

When chromaffin granules,prepared by sedimentation through 1.8 M sucrose, are lysed by freeze-thawing in the presence of 1,5-I-AEDANS and then subsequently labelled with 1,5-I-AEDANS both the membrane (see Fig. 7.5) and the lysate (see Fig. 7.3.1) show extensive labelling. The labelling pattern of the granule lysate is similar to that obtained when isolated total granule lysate was directly reacted with 1,5-I-AEDANS. In Fig. 7.3.1 is shown for comparison the incorporation of label into granule lysate when intact 'large granule fraction' granules are reacted with 1,5-I-AEDANS in the absence of freeze-thawing but otherwise labelled and treated under identical conditions. The labelling pattern of the granule membranes from this 'control' experiment is depicted in Fig. 7.5 together with that from the freeze-thawed granules. One can see that the two traces are virtually identical, the only difference being relatively more fluorescence associated with CBB stained bands 23 to 27 in the membranes prepared from freeze-thawed labelled granules. The two patterns are similar to those previously described for both granule membranes prepared from labelled granules and granule membranes labelled after isolation.

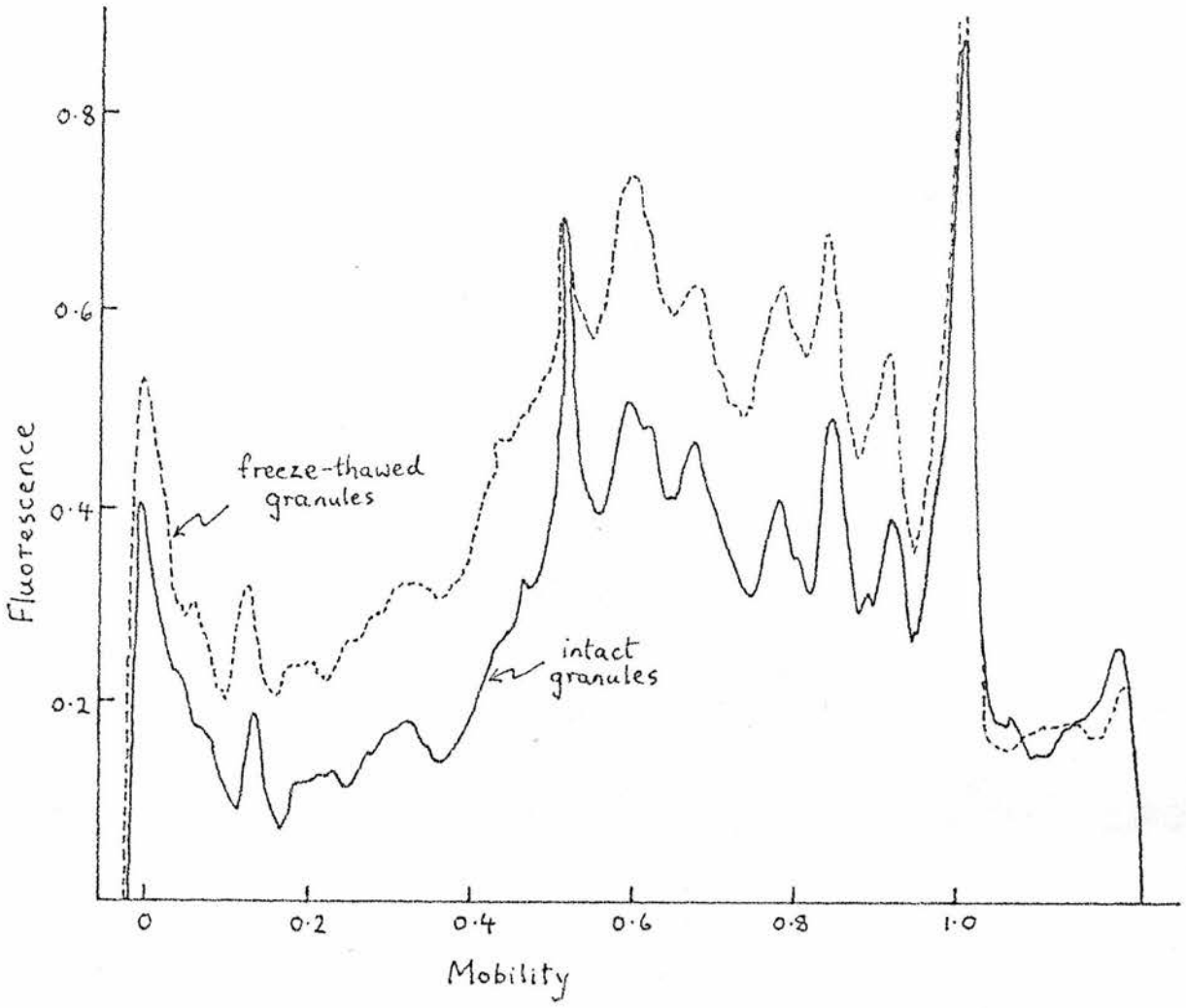

Fig. 7.5. 1, 5-I-AEDANS Labelling of Granules in the Absence and Presence of Freeze-Thawing

Intact P_2 granules or freeze-thawed granules purified by sedimentation through 1.8 M sucrose were labelled with 3 mM 1, 5-I-AEDANS at $4^{o}C$, overnight and in the dark. The broken granules were freeze-thawed in the presence of 1, 5-I-AEDANS. Membranes were purified from the labelled granules and were subjected to SDS polyacrylamide gel electrophoresis in 8% (w/v) acrylamide gels under reducing conditions. The labelling profile of each membrane preparation was determined by fluorescence scanning.

Thus we can conclude that the presence of granule membranes or any other component of the granule does not inhibit the labelling of the granule matrix proteins and it would appear that it only needs disruption of the granule membrane as a permeability barrier for extensive labelling of lysate proteins to take place when 1,5-I-AEDANS is added to chromaffin granules. However, despite labelling of the matrix proteins, membranes prepared from I-AEDANS labelled freeze-thawed granules show a similar pattern of labelling to membranes prepared from intact granule labelled with I-AEDANS. Granules were freeze-thawed in the presence of 1,5-I-AEDANS to ensure that the reagent is available to the inner surface of the membrane even if the broken granules subsequently reseal. Purified granules were used in this experiment rather than the large granule fraction because damaged granules cannot be purified by sedimentation through 1.8 M sucrose.

7.4 Discussion

The chromaffin granule membrane has been successfully labelled with 1,5-I-AEDANS in chromaffin granules without labelling of proteins of the granule lysate. Proteins of the granule lysate are capable of reacting with 1,5-I-AEDANS when the isolated total granule lysate is incubated with this reagent or when freeze-thawed granules are used. On this basis therefore we must conclude that 1,5-I-AEDANS does not penetrate the granule membrane in the intact granule since we would expect any 1,5-I-AEDANS that did gain access to the granule matrix to react with the proteins of the granule lysate.

However, it does not seem to matter whether the membrane is acting as a permeability barrier or not to 1,5-I-AEDANS since the labelling pattern of the proteins of the granule membrane is the same whether intact granules or purified granule membranes are used. This is unex-

pected since we would expect additional membrane proteins, i.e. those proteins exposed to the matrix of the granule, to be labelled when purified membranes are reacted with 1,5-I-AEDANS. If we accept that 1,5-I-AEDANS cannot get inside intact granules then this observation can be accounted for by several possibilities, the first of which is that all accessible sulphydryl groups of the chromaffin granule membrane are located on the outer surface of the chromaffin granule membrane only. In this case, exposure of the inner surface of the granule membrane would not produce extra reactive sites for 1,5-I-AEDANS. The second possibility is that purified granule membranes all vesiculate and that all the vesicles produced have their membranes orientated in the same way as intact granules; 1,5-I-AEDANS might not be able to penetrate such vesicles. It seems unlikely that all the purified membrane fragments would reseal the same way round and, in any case, this explanation appears to have been ruled out: membranes prepared from granules freze-thawed in the presence of 1,5-I-AEDANS show a similar labelling pattern to purified membranes reacted with 1,5-I-AEDANS. Under these labelling conditions 1,5-I-AEDANS definitely has access to the inner surface of the granule membrane. Thus, unless we assume that all available sulphydryl groups are present on the outside of the granule, we must question the validity of the use of labelling of the granule matrix proteins as a control for the permeability of this compound and suggest that the reagent 1,5-I-AEDANS does penetrate the membrane and consequently labels both sides of the chromaffin granule membrane in intact granules.

We are left, therefore, to answer the question of why 1,5-I-AEDANS fails to react with the lysate proteins when both are present inside the granule whereas when both are outside the granule they clearly react together. Chromaffin granules have an internal pH of between 5 and 5.5. Perhaps 1,5-I-AEDANS will not react at pH 5. It is unlikely

that there is an inhibitor present in the granule matrix since the lysate proteins are labelled in freeze-thawed granules. In any case, in both these situations labelling of the inner surface of the chromaffin granule membrane should also be inhibited so that we would have observed differences in the labelling of the membrane proteins in the granule and membrane states. The only explanation that would allow for 1,5-I-AEDANS inside the granule to react only with the inner surface of the granule membrane and not the granule lysate is that the conformation of the granule matrix proteins(see Introduction) makes their sulphydryl groups inaccessible to 1,5-I-AEDANS. When these proteins were labelled by freeze-thawing granules in the present of 1,5-I-AEDANS their conformation would probably be altered; the native conformation is likely to be dependent on the concentration, pH and other conditions existing within the matrix.

Thus we may conclude that either all the reactive sites of 1,5-I-AEDANS in the granule membrane are on the outside of the granule or that 1,5-I-AEDANS is membrane penetrant but fails to react with the matrix proteins in intact granules. It is not possible to distinguish between these possibilities. If we speculate that I-AEDANS is membrane penetrant then it would appear that the majority of the granule membrane proteins are exposed at one or other of the surfaces of the membrane (assuming that sulphydryl groups are likely to be at the surfaces of membranes). Furthermore these results would indicate that the conformation of the granule membrane is the same in granules and isolated membranes. It has been suggested that membranes undergo structural rearrangement upon lysis and/or subsequent purification (see Introduction).

CHAPTER 8

CONCLUSION

CONCLUSION

Chromaffin granule membranes have been separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis under reducing conditions. Almost complete solubilization of the membrane was achieved and at least 66 bands could be detected in the gels by staining with coomassie brilliant blue. This number of bands is probably an underestimate of the number of different polypeptides in the membrane because of the real possibility that each band may contain more than one polypeptide and is a series problem when analyzing the results of the labelling experiments. The majority of the bands are minor staining components. The major staining bands have been identified as the subunits of the enzyme dopamine-β-hydroxylase whilst bands 28, 29 and 44 may represent the subunit of the Mg^{++} ATPase. The second major stained band has been referred to as chromomembrin B (Winkler, 1971) although my results indicate that there are, in fact, at least 3 bands in this region.

The granule membrane also contains a number of glycoproteins which have been detected by staining gels with periodate - Schiff reagent, dansyl hydrazine and fluorescein isothiocyanate conjugated concanavilin A and wheat germ agglutinin. Galactose oxidase or periodate treatment of the membranes followed by reduction with [^{3}H]borohydride has also been used to identify glycoproteins. Ten glycoproteins have been identified 6 of which are major and 4 minor staining components. The major glycoproteins can be detected by most if not all of the methods listed above. Dopamine-β-hydroxylase is one of the major glycoproteins. Chromomembrin B does not appear to be a glycoprotein.

Galactose oxidase or periodate treatment followed by reduction with [^{3}H]borohydride can also be used to determine which side of the granule membrane the carbohydrate moieties of the glycoprotein are exposed at. Glycoproteins could only be labelled when broken membranes were used indicating that all protein bound carbohydrate is exposed to

the matrix of the granule. Thus if the plasma membrane of the chromaffin cell is similar in basic structure to other plasma membranes which have been characterized then any fusion between the plasma membrane and granule membrane will result in the maintainance of the asymmetry of the plasma membrane. It does, however, seem that the carbohydrate moieties of the granule membrane glycoprotein cannot be involved in or act as receptors for the initial contact phases of exocytosis.

The topography of the membrane glycoproteins has also been determined by proteolytic digestion with pronase. One glycoprotein (GPIb) was degraded when intact granules were digested. Since we know that the carbohydrate moiety of this glycoprotein is exposed on the matrix side of the membrane then it would appear that GPIb spans the membrane. Two other glycoprotein(DβH and GP VII)were degraded when isolated membranes were digested confirming their localization on the matrix side of the membrane.

Of the 45 CBB bands of granule membranes which are susceptible to pronase digestion, 38 are sensitive in intact granules. DβH can only be digested in broken membranes whilst chromomembrin B can be digested from both sides of the membrane and probably spans the membrane. Enzyme assays confirmed that DβH is only attacked in broken membranes but showed that phosphalidylinositol kinase, Mg^{++} ATPase and NADH:(acceptor) oxidoreductase are all exposed on the outside of the granule.

The majority of the chromaffin granule membrane polypeptides could also be iodinated following treatment of intact granules with lactoperoxidase and ^{125}I. Of particular note was the very heavy labelling of a small molecular weight component (band 49) thought to comprise the staining region referred to as chromomembrin B. Other bands in the chromomembrin B region (bands 50 and 51) were also labelled in granules. Dopamine-β- hydroxylase, once again, could only be significantly iodinated when broken membranes were used. Relatively few bands were iodinated

in broken membranes but not granules. However, several bands showed increased labelling when broken membranes were labelled compared to when granules were used suggesting that GPIb and chromomembrin B may not be the only membrane proteins that span the membrane.

Although N-(iodoacetylaminoethyl)-5-napthylamine-1-sulphonic acid (1,5-I-AEDANS) did not label intra-granule proteins when intact granules were labelled, indicating this reagent does not penetrate the membrane, labelling of broken membranes and intact granules produced the same pattern of labelling of the membrane components. Virtually all the major CBB staining bands, with the exception of DβH, were labelled with 1,5-I-AEDANS.

Thus we may conclude the following about the arrangement of the proteins of the chromaffin granule membrane: the majority of the polypeptide species of the membrane are located on the cytoplasmic suface of the membrane whereas the carbohydrate moieties of the membrane glycoprotein are exclusively located on the matrix side of the membrane: dopamine-β-hydroxylase is present only on the matrix side whereas the other 3 membrane-bound enzymes, phosphatidylinositol kinase, Mg^{++} ATPase and NADH: (acceptor) oxidoreductase are exposed to the outside of the granule: chromomembrin B and GPIb span the membrane although this is an underestimate since in these studies transmembrane proteins would be assigned as being located on the cytoplasmic face of the membrane.

These features are consistent with the expected asymmetric structure of organelle membranes and would allow fusion of the granule membrane and plasma membrane to maintain the asymmetry of both membranes. Of interest is the confirmation of the finding that chromomembrin B is prominently located on the outside of the granule (Konig et al., 1976).

Winkler (1974) believes that chromomembrin B, which is unique to the chromaffin granule membrane, may be involved in the initial contact phase of exocytosis. Being a prominently exposed and relatively abundant protein it would contribute significantly to the carboxyl groups present on the outside of the granule which make up the charge on the granule. Calcium binding would neutralise this charge and concomitant neutralization of the charge on the inner surface of the plasma membrane could allow the two membranes to approach close enough to fuse.

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